MASTER'S THESIS IN MOLECULAR BIOLOGY

Mapping of Bacteria with Flow Cytometry

- before, during and after treatment of drinking water

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60 credits

Conducted in Görvälnverket,
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Abstract

In order to produce drinking water of high quality, surface water works apply extensive treatment processes. In this study, flow cytometry and heterotrophic plate counting was utilized to study water quality before, during and after treatment in Görvälnverket in Stockholm, Sweden. Samples were collected and analysed throughout the treatment process, mapping the reduction of total and intact cell counts as well as bacteria possessing high nucleic acid content, which is a characteristic of some pathogens. Total and intact cell counts decline throughout autumn and stabilize at the end of December. At the same time, the number of bacteria with high nucleic acid content increase, possibly displaying circulation in the lake and therefore intake of sediment bacteria. The treatment of flocculation and sedimentation decrease bacterial counts to a large extent. Samples taken after sand filtration, carbon filtration and finished drinking water display similar counts. According to this data, the number of effluent bacteria from sand filters depend heavily on maintenance timing. Carbon filtration does not seem to alter the numbers of bacteria. Lastly, biostability was evaluated and compared to commercially sold bottled water. Norrvatten's produced drinking water yield the least cfu and display sufficient biostability. The reason seems to be monochloramine addition and UV radiation that disinfects and inhibits bacterial regrowth, respectively.

Introduction

Görvälnverket is a Swedish surface water works located in Stockholm with its primary water source from lake Mälaren. Norrvatten owns the water works and distributes drinking water to 600 000 people. It has an average capacity of 140 000 m^3 water per day along its 320-km long distribution system. There are 8 functioning water reservoirs around Stockholm. Production of drinking water is done in multiple steps according to Figure 1. First the lake water is sieved (200 µm) from fish, algae and other large objects (Norrvatten). Then the coagulant aluminium sulphate is added to bind humus, microorganisms and other particles. Sodium silicate is added to increase the size of the bound particles and make the created flocks more stable. Through sedimentation these impurities are removed. This barrier that separates through chemical precipitation and sedimentation consists of 2 different processes in Görvälnverket: Separately in 2 different water tanks and in a high-technological combination-process called the pulsator. Both these methods are in service simultaneously. Thereafter the water pass through sand filters (18 individual) to remove additional aggregated organic material and microbes. The water passing through individual filters is collected for continued processing. Additional factors other than the status of lake Mälaren affect the results of an individual sand filter, such as sand thickness and time since the last backflush. Then filters with active granulated carbon are used to absorb and therefore reduce the amount of organic compounds in effluent water that could otherwise result in odor and taste of the water (Scott & Pepper 2010; Velten et al 2007; Servais et al 1991). Carbon filters at Görvälnverket are usually backflushed every 336th hour (Norrvatten). After filtering, the water passes through ultraviolet light, which is a second microbiological barrier that damages DNA of remaining microorganisms. UV treatment is a highly useful disinfection technique in water works because it does not involve any addition of chemicals (Madigan et al 2012). Before distributing the water to customers, low concentrations of agricultural lime and monochloramine are added to adjust pH and inhibit growth of microorganisms, respectively. This is done because microbes can cause corrosion in distribution systems (Wang et al 2017). There are a few aspects in the microbiological perspective of drinking water treatment. Most important is to ensure total absence of pathogenic bacteria (Hammes et al 2008). It is therefore important to analyse raw water quality, along production and distribution systems for potentially harmful microbes (WHO 2008). The treatment process is illustrated in Figure 1 and more information about it can be found in supplementary materials.

Traditional heterotrophic plate counting is one of the standard routine microbial analysis in water works. Positive results are certain proof of viability for those cells. It is cost-friendly and there is a lot of historic data that can be used to compare. A few downsides are the amount of work and time from sampling to results (van Nevel et al 2017a). The vast majority of planktonic bacteria in drinking water are not yet cultivable. A few possibilities are that they may be uncultivable, persisters or viable but not cultivable (Hammes et al 2008; Wang et al 2009; van Nevel et al 2017a). Cultivation-dependent methods are not able to give the full picture of bacterial communities. Today the general consensus is that heterotrophic plate counting has no hygienic relevance in a water works (van Nevel et al 2017a; Allen et al 2004). However, Livsmedelsverket approves the use of yeast-peptone agar in such analyses (SS-EN ISO 6222). Therefore, it is used in this project as reference although the medium has much higher nutrient levels (approximately 800 times) than the natural environment in lakes

(Allen et al 2004). Today flow cytometry is one possible technique to analyse bacteria that are present in drinking water (Wang et al 2009). Heterotrophic plate counting displays very little, if any, correlation with flow cytometric data after staining (Siebel et al 2008). Flow cytometry has proven to be a highly useful technique in the field because it is precise and fast. Each cell passes through laser optics and scatters as well as emits fluorescent light based on the biological stain used. Several detectors in the instrument detects different events (Prest et al 2013; van Nevel et al 2017b; Bundesamt für Gesundheit). In this study, flow cytometry was applied to count and characterize bacteria based on nucleic acid content in the water at different steps in the production. There are other occasions when flow cytometry is applicable in a water works, such as necessary flushing time needed in maintenance of pipe networks (van Nevel et al 2017b). The fluorescence intensity is used to distinguish bacteria with low nucleic acid content from those with high nucleic acid content (Belzile et al 2008; Bundesamt für Gesundheit). It does not provide information of which species are analysed but nonetheless flow cytometric data gives insight into the effect of microbiological barriers in the water works (Ramseier et al 2011). It is an important factor to consider when evaluating drinking water quality (Liu et al 2013b). Plausible methods to complement flow cytometry and gain information of taxonomy of the organisms are massively parallel DNA sequencing. By performing the same routine analysis for one or several years, it is possible to create a baseline of microbial community (van Nevel et al 2017a). Measurements and standard deviations during all months show what levels of bacteria constitute normal fluctuations. This baseline can then be used as comparison to quickly detect abnormal changes of microbial community based on both total cell count and nucleic acid content composition. An increase in bacterial abundance may be a health risk (van Nevel et al 2016). Extensive growth of bacteria may also cause the drinking water to have undesired taste, odor and turbidity (Hammes et al 2008). Therefore, such swifts of bacterial community might require action in the water works (van Nevel et al 2016).

In order for the flow cytometer to accurately count and distinguish events, fluorescent staining of cells is necessary. It is versatile in the sense that different stains can be utilized in different studies to display the specific cell compartment of interest (Prest et al 2013). SYBR Green 1 staining can be applied for total cell counting as well as characterizing nucleic acid content because it stains all bacterial cells no matter the physiological state (van Nevel et al 2016; Berney et al 2008). It intercalates the minor groove of double-stranded DNA which leads to a hundred-fold more intense fluorescence and low background fluorescence (Zipper et al 2004; ScienceDirect). Propidium iodide is normally excluded from cells by the cytoplasmic membrane due to its large size (Joux & Lebaron 2000). It can therefore be added to stain cells with severe damage on the cytoplasmic membrane. This almost translates into a form of viable count although it is essential to realize the limit of such a conclusion (van Nevel et al 2016; Nescerecka et al 2016). The combination of these two stains suits the experiment well because it is possible to differentiate total cell count from intact cell count. Intact cells display green fluorescence while damaged cells display red fluorescence as well (Joux & Lebaron 2000). It is essential that the staining procedure is easily repeatable and it may be automated in the future to enable online data comparison worldwide (Besmer et al 2014).

Characterization of bacteria based on nucleic acid content is an ongoing area of research. Bacteria with high nucleic acid content (HNA) are distinguished from bacteria with low nucleic acid content (LNA) by an artificial threshold, described by Prest et al 2013. The clusters of HNA and LNA bacteria are after staining of nucleic acids separated by fluorescence intensity and possibly light scattering (Proctor et al 2018). Most studies indicate that HNA microorganisms are more metabolically active and dynamic than LNA (Wang et al 2009; Belzile et al 2008; Lebaron et al 2001; Gasol et al 1999). HNA and LNA bacteria are different organisms according to sequencing studies (Proctor et al 2018; Wang et al 2009). LNA bacteria seem to be represented by previously characterized ultramicrobacteria according to sequencing (Proctor et al 2018). HNA bacteria on the other hand are represented by the most commonly known bacteria and known pathogens are in this category (Ramseier et al 2011). The variability of HNA bacteria seems to be heavily dependent on nutrient availability, LNA bacteria numbers on the other hand are not. Therefore, the numbers of HNA and LNA bacteria will vary depending on season (Liu et al 2016) and nutrient levels due to human activity (Harry et al 2016).

Freshwater systems like lakes can be described as abiotic frames. Each one has physical and chemical characteristics that govern biology. The most important and obvious factors are temperature, pH, oxygen levels, nutrient concentrations and light availability. Furthermore, biotic interactions affect the organisms in the lake. Examples are competition, mutualism, parasitism and predation (Brönmark & Hansson 2017). There are seasonal variations that affect the ecology of lakes in many different ways. According to SMHI, climate change will affect this cycle in lake Mälaren (Stensen et al 2017). The annual time that ice covers the lake will be reduced by 1 – 2 months by year 2100. Mean surface and bottom temperature will increase 1-5 – 2.5°C and 1 – 2°C respectively. The maximal temperature will increase more than mean temperature. Temperature is an important factor for the efficiency of biological processes (metabolic rates) (Brönmark & Hansson 2017) because most aquatic organisms are poikilothermic, possessing variable temperature according to surroundings. Most organism's metabolic rates increase in higher temperature, and therefore also growth rates (Tikkanen & Willén 1992). Total cell count measured with flow cytometry reveals higher numbers in temperatures above 15°C than below (Liu et al 2013b). It is important to point out that temperature is not the only factor in microbial growth, nutrient levels like phosphorous and nitrogen are also of great importance (Willén 2001; Tikkanen & Willén 1992; Perlman 1977). In lakes the number of virus is approximately 10^7 while prokaryotes and eukaryotes like fungi and protozoa are usually on a 10^6 scale or less (Brönmark & Hansson 2017). Numbers of bacteria are reduced by grazers and infecting phages (Perlman 1977).

In order to evaluate the drinking water quality of Norrvatten, one can compare it to national/international guidelines and bottled water for sale. WHO's international guidelines for drinking water is 20 cfu/ml and 300 cfu/ml for distributed water (WHO 2006) although national rules are often much stricter. Comparison can be done using both methods: heterotrophic plate counting and flow cytometry.

This full-scale study has several objectives. First of all, flow cytometry and heterotrophic plate counting were used to monitor the bacterial dynamics throughout the entire study period of five months regarding total cell count, viable cell count and number of HNA. Water from the pulsator was compared to water that went through flocculation and sedimentation steps. The individual sand filters and carbon filters were evaluated based on maintenance optimization. A comparison was performed on produced drinking water and various bottled drinking water sold commercially. Lastly, the long-term effects of UV radiation and/or monochloramine were determined.

Materials and methods

Collection of samples

Water samples were collected from 4 chosen sampling points in the water works in the morning 3 times a week. These sampling points were kept throughout the experiment: Raw water (R), after sand filtration (SF), after carbon filtration (CF) and finished outgoing drinking water (DW). Water samples were collected in sterile sodium thiosulfate (STS) bottles and used within 3 hours. STS absorbs chlorine, which is necessary to obtain accurate viable count values (WHO 1963). Triplicates were run for each water sample to increase statistical significance and test repeatability of the method (Prest et al 2013).

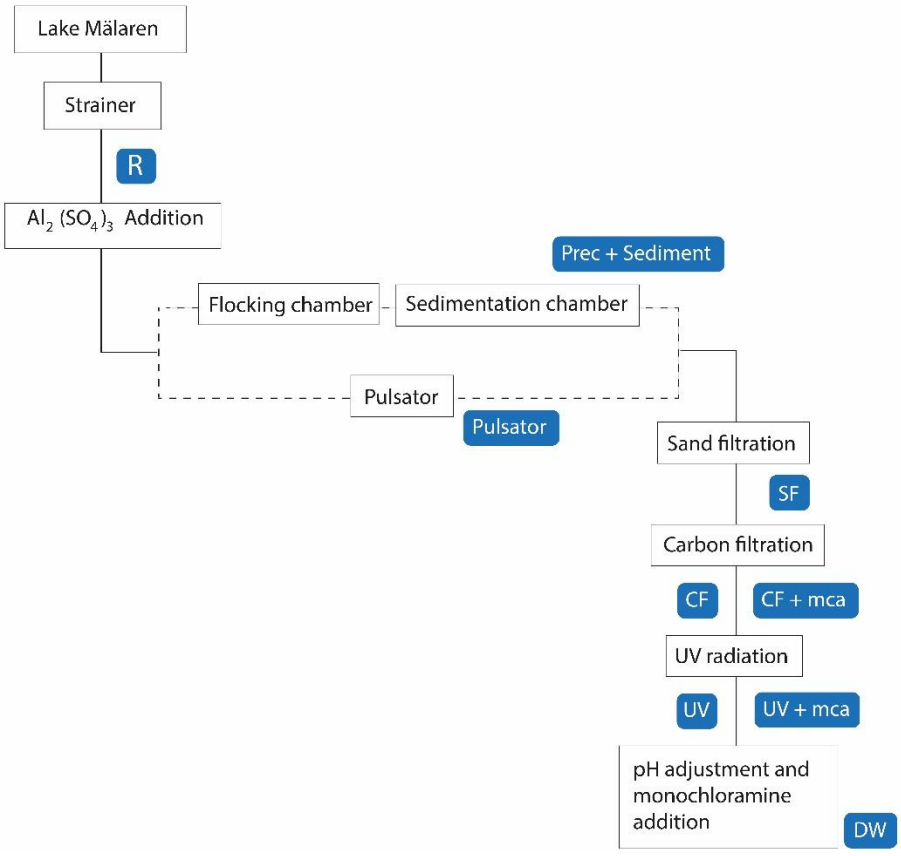


Figure 1: Overview of the drinking water process from lake to customer. Treatment steps have white backgrounds and collected samples have blue backgrounds. Abbreviations are: R

= raw water, SF = effluent sand filtrated water, CF = effluent carbon filtrated water, UV = water sampled after UV radiation, DW = finished drinking water ready for distribution.

Routine analysis procedure

Heterotrophic plate counting was performed on cultivation plates containing enzymatic digest of casein (6 g/l), yeast extract (3 g/l), agar (15 g/l) and water. That way the baseline of microbes is highly comparable between analysis methods. Both 3- and 7-days growth analysis was done according to the routine at Norrvatten that follows the standard method SS-EN ISO 8199:2007. After 3 and 7 days respectively, the number of colony forming units (cfu) were counted in a stereomicroscope (Olympus SZX7) with 10 times magnification. Flow cytometry was performed with a BD Accuri C6 Plus (BD Biosciences). It is equipped with a red (640 nm wavelength) and a blue laser (488 nm wavelength), two detectors for light scattering and four detectors for fluorescence that are optimized for popular fluorochromes. Data collection was done via FL1/FL3 channels of green/red fluorescence dot plots that allow distinction between cell counts, sample background and instrument noise (Hammes et al 2008). Background signals are caused by salts, cell fragments, virus and free DNA to name a few (van Nevel 2017b). Gating was performed according to instructions (Prest et al 2013) to create a solid template used for all samples throughout the experiment. Staining of water samples was performed according to guidelines (Nescerecka et al 2016; Bundesamt für Gesundheit). Prior to flow cytometric analysis, stains (SYBR Green 1 and propidium iodide, Invitrogen) were added to samples, vortexed (Vortex-Genie 2, Scientific Industries) and allowed to incubate for 15 minutes at 35 ± 2°C in the dark. It is critical that the staining procedure is consequently done in the same reproducible way (Prest et al 2013). Working stock solution of SYBR Green was prepared by diluting it 100 times in dimethylsulphoxide (DMSO). Working stock of PI/SYBR Green mixture was prepared by blending 21 µl of PI concentrate and 105 µl of SYBR Green working stock. The samples for total cell count were run with 5 µl of SYBR Green added to 495 µl water sample. The samples for viable cell count were run with 6 µl PI/SYBR Green mixture and 494 µl of the water. SYBR Green was used to stain both negative controls (autoclaved Milli Q water) and positive controls (*Escherichia coli* in buffer). The buffer contained Milli Q water, KH_2PO_4 and $MgSO_4$.

Bottled and produced drinking water management

Two different brands of bottled water were bought from a local supermarket and stored dark and cold (4°C) throughout the 24-days study. As comparison, produced drinking water was collected the same date in sterile STS bottles and a sterilized glass bottle. These 4 different samples were run at least once every week in the flow cytometer in parallel with cultivation on nutrient plates. Each bottle was thoroughly shaken before usage.

Obtaining samples of monochloramine and UV radiation

Samples exposed to all 4 combinations of monochloramine and UV radiation had to be obtained in the water works. These samples were: Lack of both treatments, UV radiation, monochloramine and finally a sample exposed to both treatments. CF water lacks both. A

sampling point in the water works tapped out CF water after UV radiation. A different sampling point gave water exposed to both UV radiation and monochloramine, differing to DW in the aspect that it has not been pH adjusted. Finally, CF water was taken and manually mixed with similar concentration of monochloramine as in production (0.23 mg/l). All 4 samples collected/created were stored in sterile glass bottles and shaken thoroughly before usage.

Results

Raw flow cytometric data consists of dot-plots over a gate of choice. This quantifies many aspects such as total cell count, instrument noise and nucleic acid intensity due to staining. The gate of choice is for bacteria solely. The artificial threshold between HNA and LNA is illustrated in Figure 14 in the Supplementary information, a green fluorescence histogram. The threshold is set at the same value every time (Prest et al 2013).

Figure 2 shows the progress of total cell count for the water types raw water (R), carbon filtrate (CF), sand filtrate (SF) and drinking water (DW) between October 2017 and March 2018. This is part of the study to create a bacterial baseline throughout the year to gain understanding about normal bacterial levels. The processed water types are very cohesive throughout and are fairly stable at 550’000 cells/ml ± 10’000 after December 18th. Raw Water displays similar pattern of stabilizing at the same date, although with much higher cell counts, approximately 1.8 million ± 12’000. Prior to sampling the 14th of February, the raw water intake conduit was switched to 4 meters of depth in contrast to 22 meters previously. This switch is marked with a red vertical line in all figures.

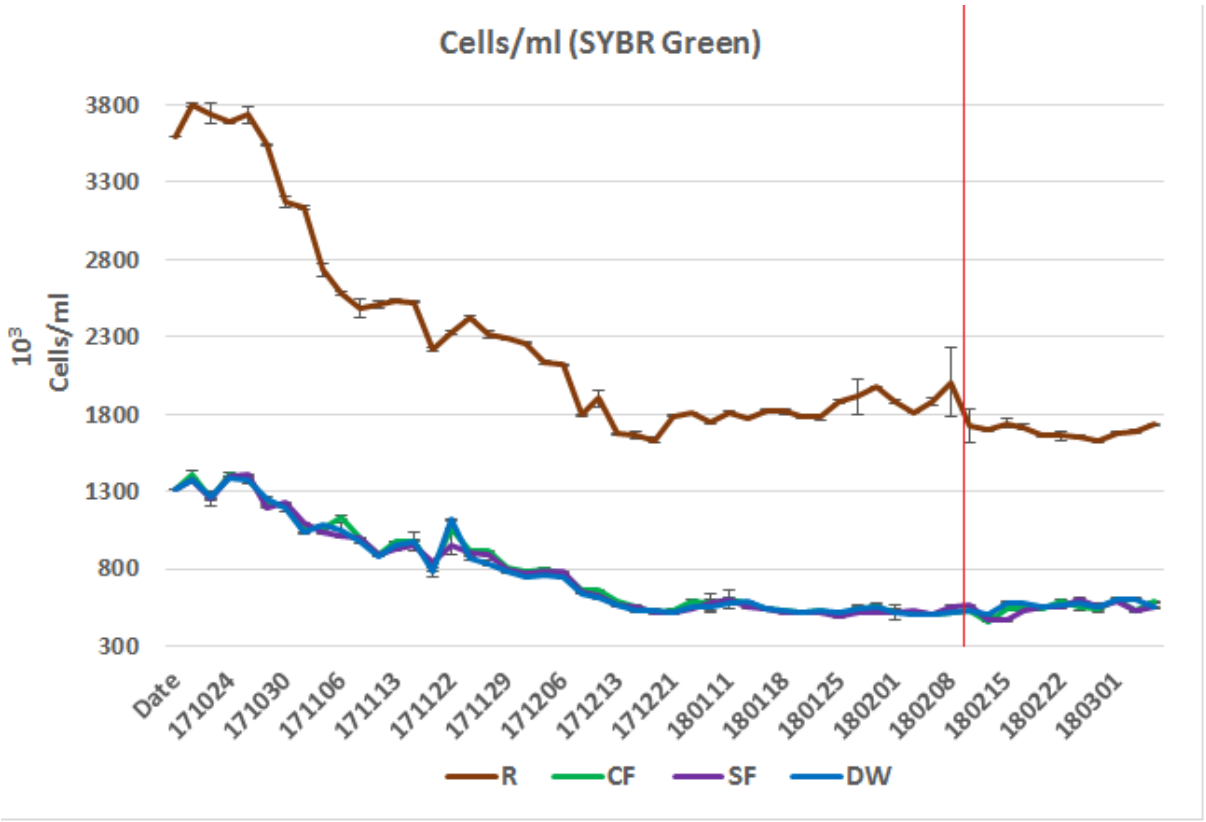


Figure 2: SYBR Green 1 total cell counts over time from the 4 different water types. Raw water, after carbon filtration, after sand filtration and finished drinking water. The vertical red line marks the switch of intake conduit depth from 22 to 4 meters. Error bars show standard deviation among triplicates.

Figure 3 shows the same as Figure 2 but report the number of intact cells. Similar pattern can be seen, bacterial counts stabilize fairly well at the end of December. Raw water stabilizes at approximately 900'000 cells/ml ± 8000. The processed samples stabilize at 300 000 cells/ml ± 15'000. Standard deviation is proportionally significantly larger using propidium iodide compared to SYBR Green.

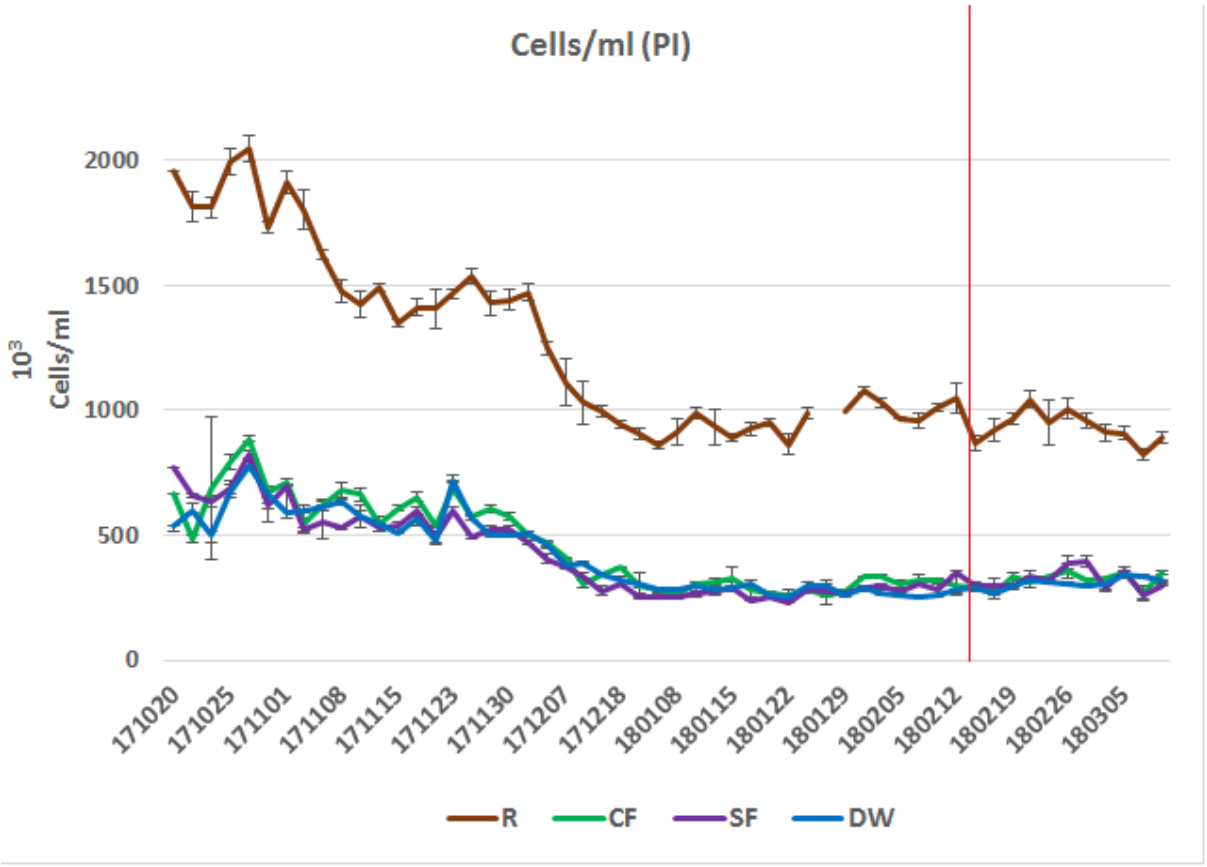


Figure 3: Propidium iodide total cell counts over time from the 4 different water sampling points in the water works. Raw water, after carbon filtration, after sand filtration and finished drinking water. The vertical red line marks the switch of intake conduit depth from 22 to 4 meters. Error bars show standard deviation among triplicates.

Figure 4 shows the same data using a bar graph for the routinely analysed raw water. This figure clearly shows the proportions of intact and damaged cells. Typically, approximately 55 – 60 % of cells were intact. Also, the temperature of raw water measured by Norrvatten is included.

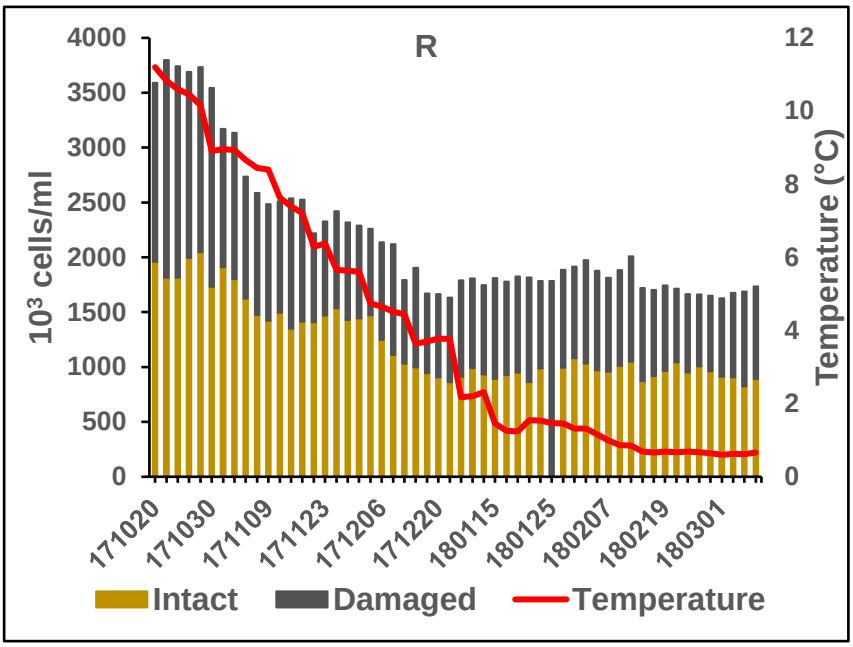


Figure 4: Bar graph showing raw water bacterial counts over time. Intact and total cell numbers are shown as well as their relative proportion. The red line shows Norrvatten's measured temperature for the analysed raw water.

According to Belzile et al, the standard error of flow cytometry sub-samples is <7 %. In this project the standard error with SYBR Green 1 was typically <2 % with a few exceptions (data not presented). Propidium iodide staining usually gave a higher standard error of approximately 7 % (data not presented). In comparison, the standard error for sub-samples in heterotrophic plate counting usually varied between 20 – 40 %.

The number of cells with high nucleic acid content varied as presented in Figure 5. The number of HNA bacteria is important to analyse because this group contains more active bacteria including potential pathogens. The lowest numbers were observed at the end of December. This was most apparent in Raw water which varied between 260'000 and 490'000 bacteria. The other water types displayed similar patterns, varying between 60'000 and 130'000 bacteria. Figure 6 show propidium iodide data instead of SYBR Green. It displays the same pattern.

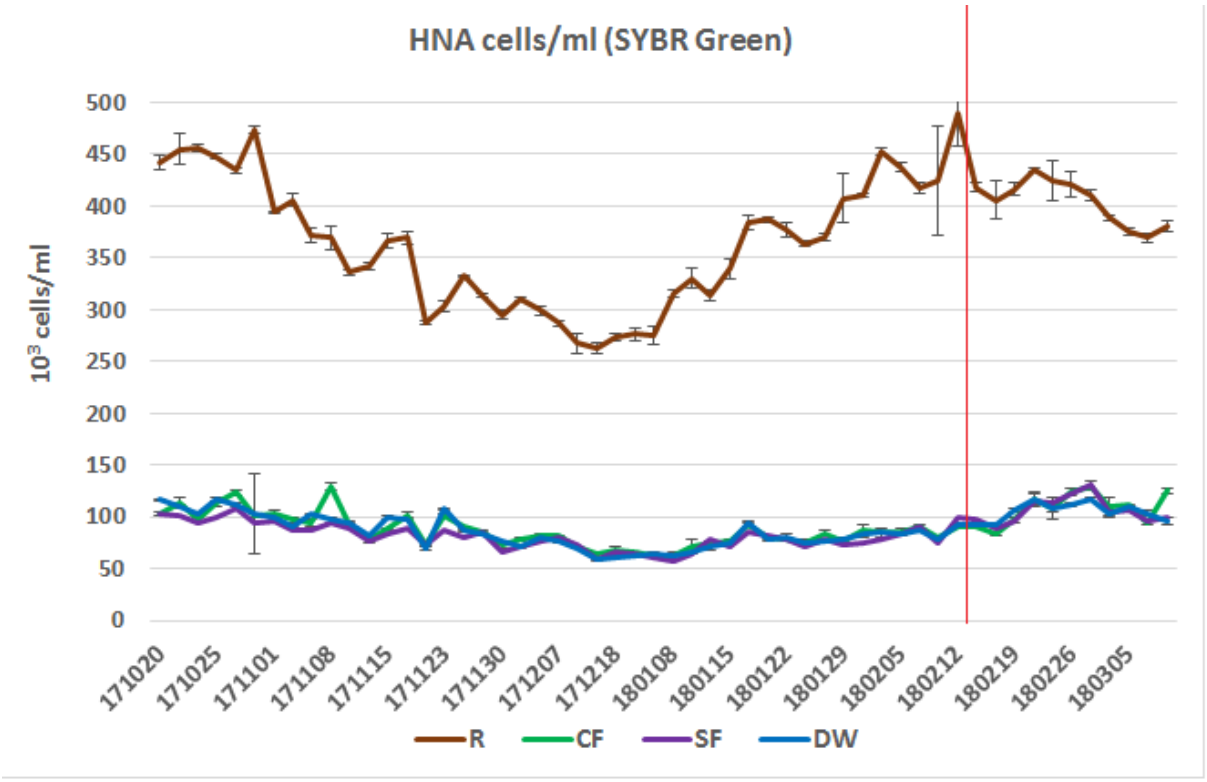


Figure 5: Number of HNA cells/ml measured with SYBR Green. Raw water, after carbon filtration, after sand filtration and finished drinking water. The vertical red line marks the switch of intake conduit depth from 22 to 4 meters. Error bars show standard deviation among triplicates.

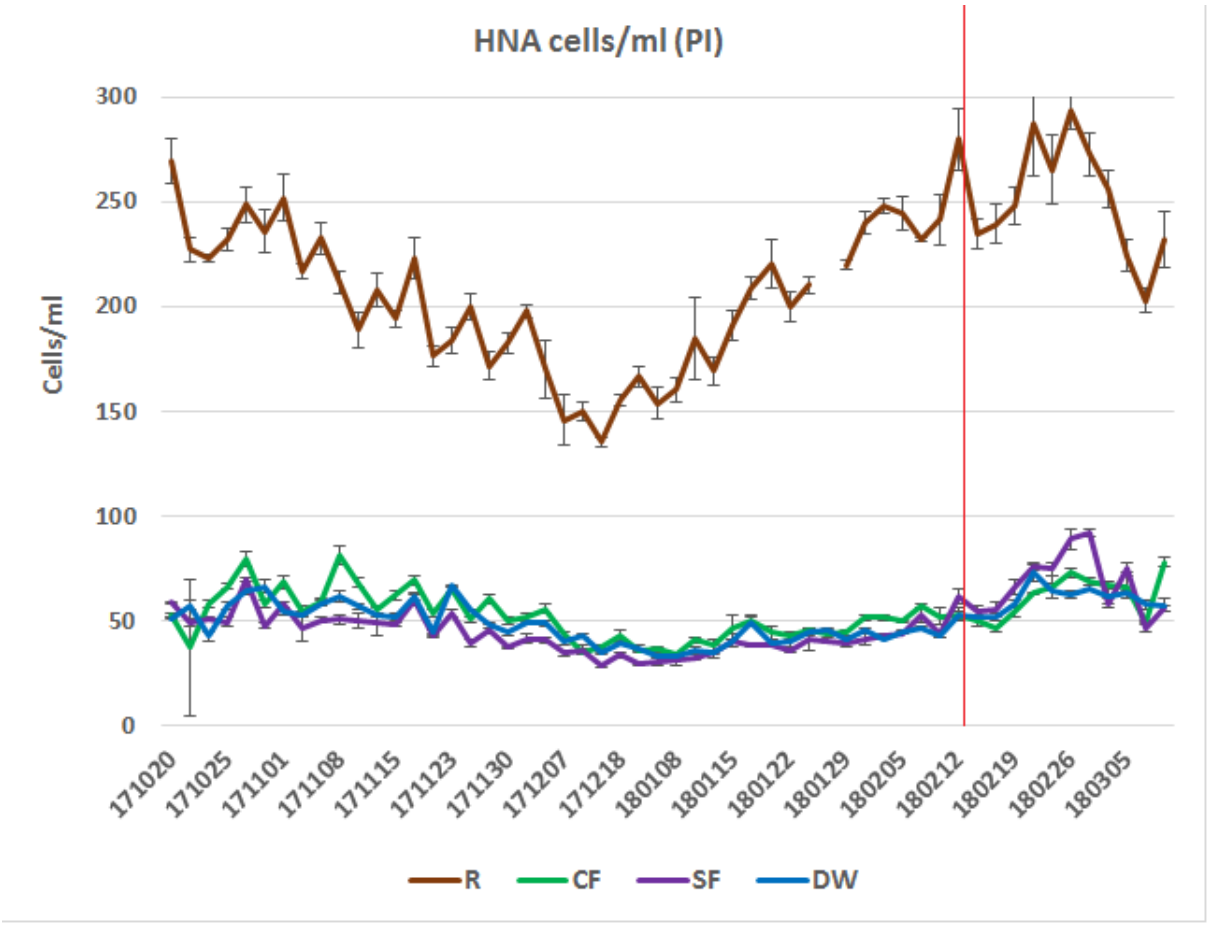


Figure 6: Number of HNA cells/ml measured with propidium iodide. Raw water, after carbon filtration, after sand filtration and finished drinking water. The vertical red line marks the switch of intake conduit depth from 22 to 4 meters. Error bars show standard deviation among triplicates.

Figure 7 and 8 show data for heterotrophic plate counts; 3 and 7 days incubation respectively. These are the standardized incubation times to count cultivable and slow-growing bacteria respectively. Growth in DW was not common although November 22nd, December 18th and January 29th gave means of 2 - 4 cfu/ml. Raw water gave the highest numbers of cfu, requiring a separate Y-axis in order to be presented in the same graph. SF and CF gave fairly similar values throughout.

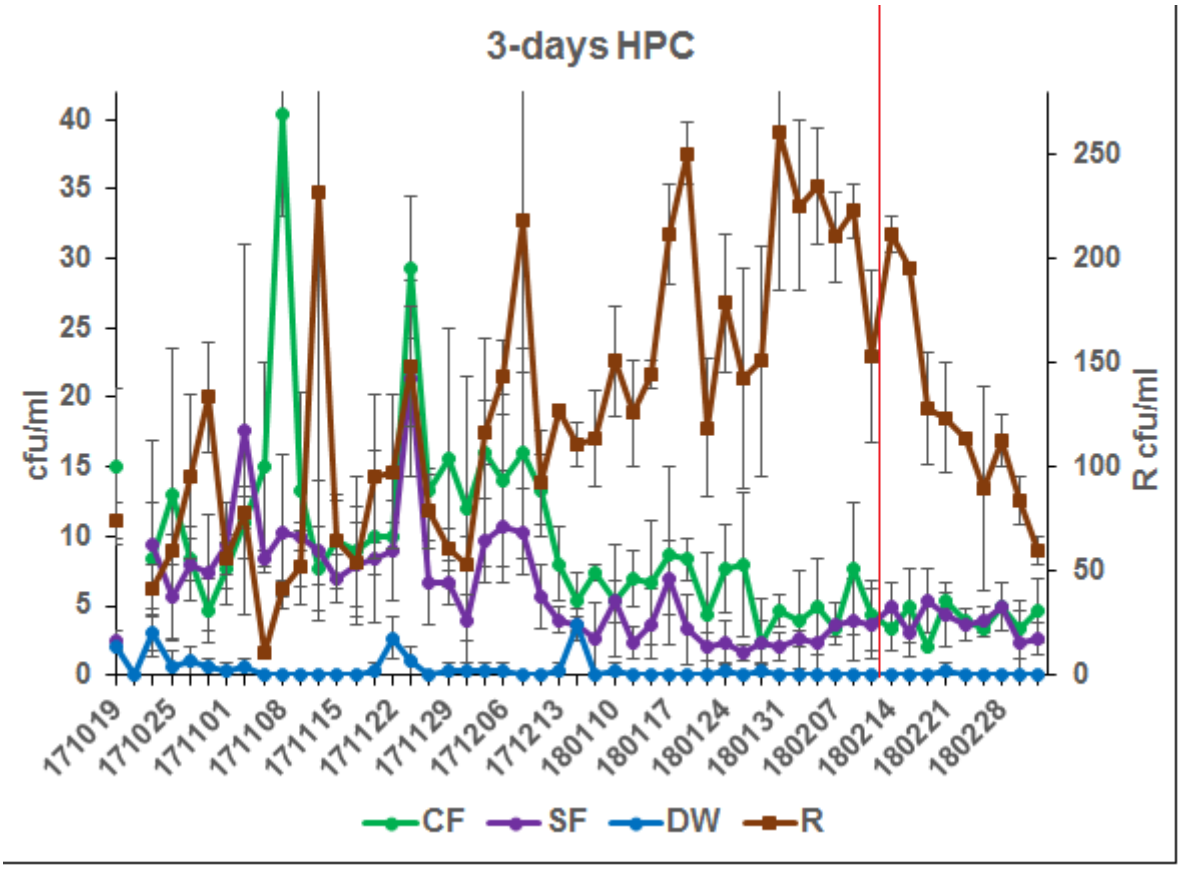


Figure 7: Heterotrophic plate counts after 3 days of incubation. Raw water, after carbon filtration, after sand filtration and finished drinking water. The secondary Y-axis is for raw water. The vertical red line marks the switch of intake conduit depth from 22 to 4 meters. Error bars show standard deviation among triplicates.

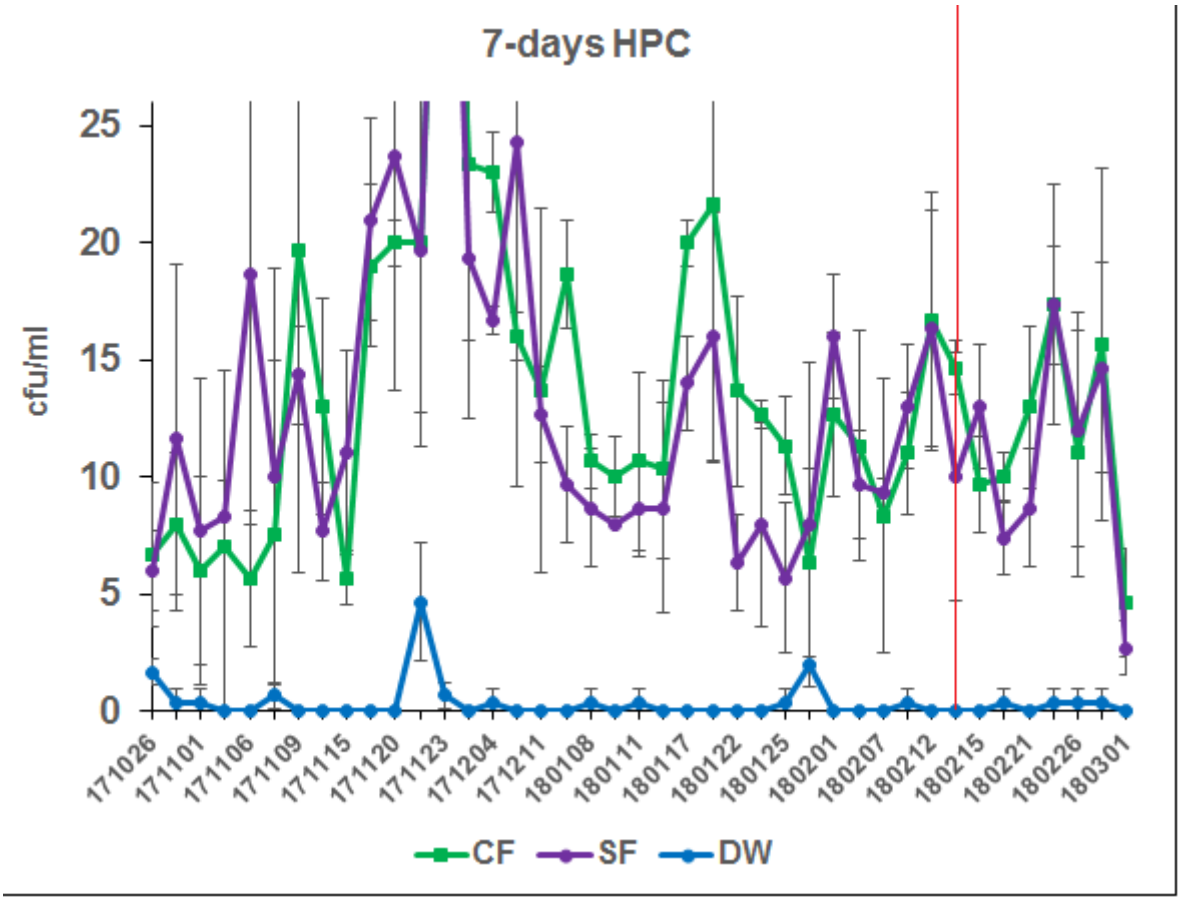


Figure 8: Heterotrophic plate counts after 7 days of incubation. Raw water, after carbon filtration, after sand filtration and finished drinking water. The vertical red line marks the switch of intake conduit depth from 22 to 4 meters. Error bars show standard deviation among triplicates.

Individual sand- and carbon filters were evaluated based on time passed since last backflush. This analysis is important to gain knowledge about optimization of maintenance timing. The mean of triplicates of flow cytometric data were divided by the collected filter values the same day. These data are presented in Figure 9 and 10. Ratio 1 on the Y-axis means that the individual filter is similar to the collected filter that day. >1 means the individual filter gives higher cell counts than the average and <1 is lower cell counts than the average. In the case of SF, a significant decrease in cell counts can be observed the longer time since last backflush. This applies to all parameters tested, total cell count, intact cell count and number of HNA cells for both types of staining. All the 14 tested sand filters were cohesive, none of them deviated from the pattern (data not presented). The 10 CF did not show such a correlation in any of the aspects total-, intact cell count or HNA. The outliers around 100 – 150 hours come from different filters and therefore not suggesting any specific filter to deviate from others (data not presented). Sand filter data was collected during December and January while carbon filter data was collected during February.

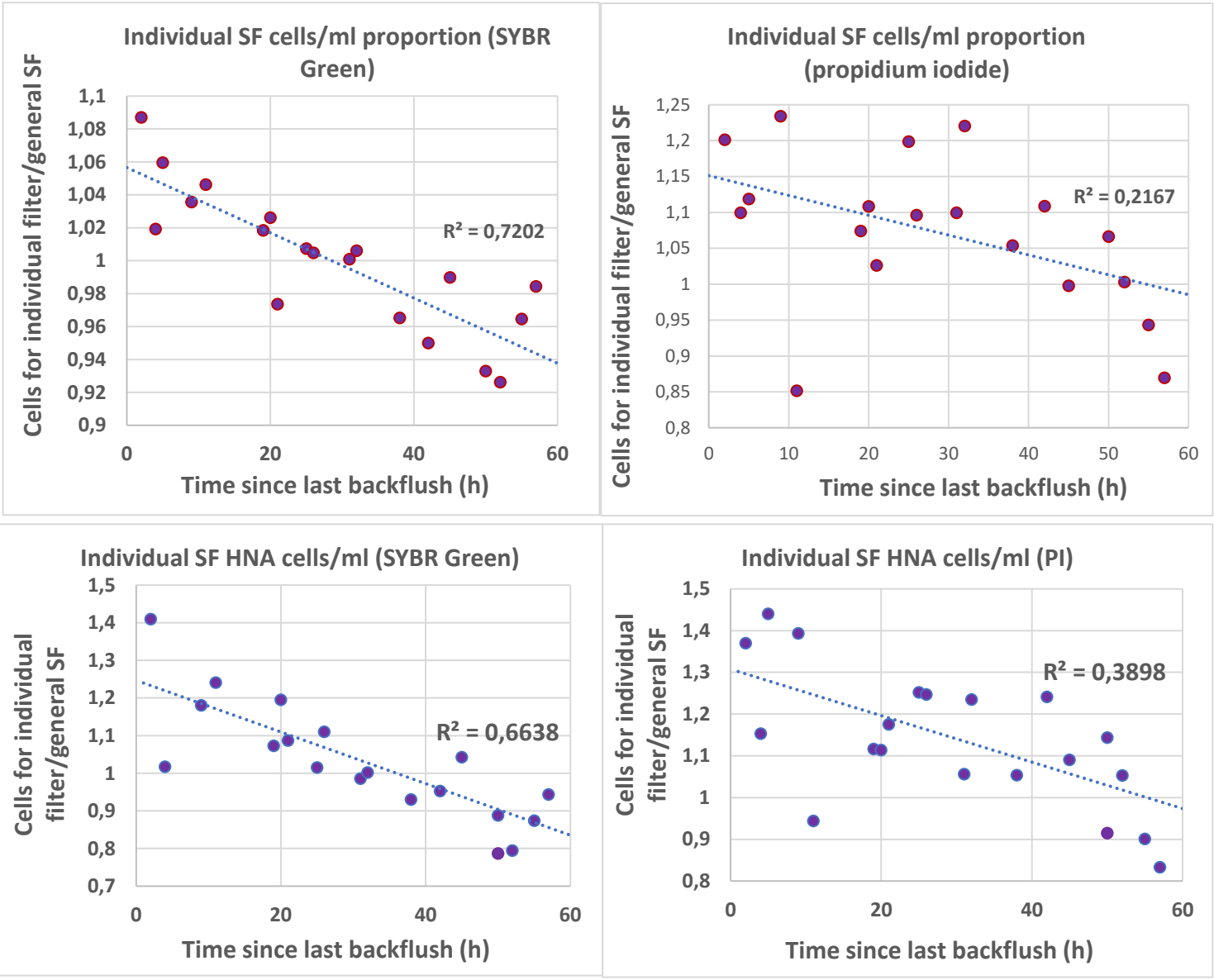
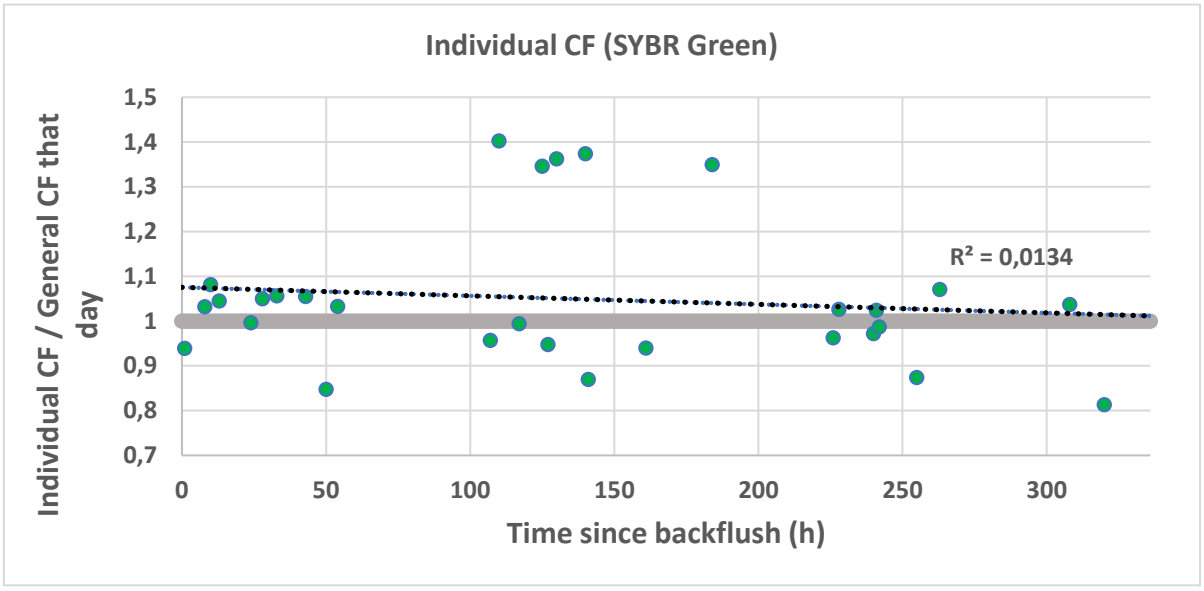
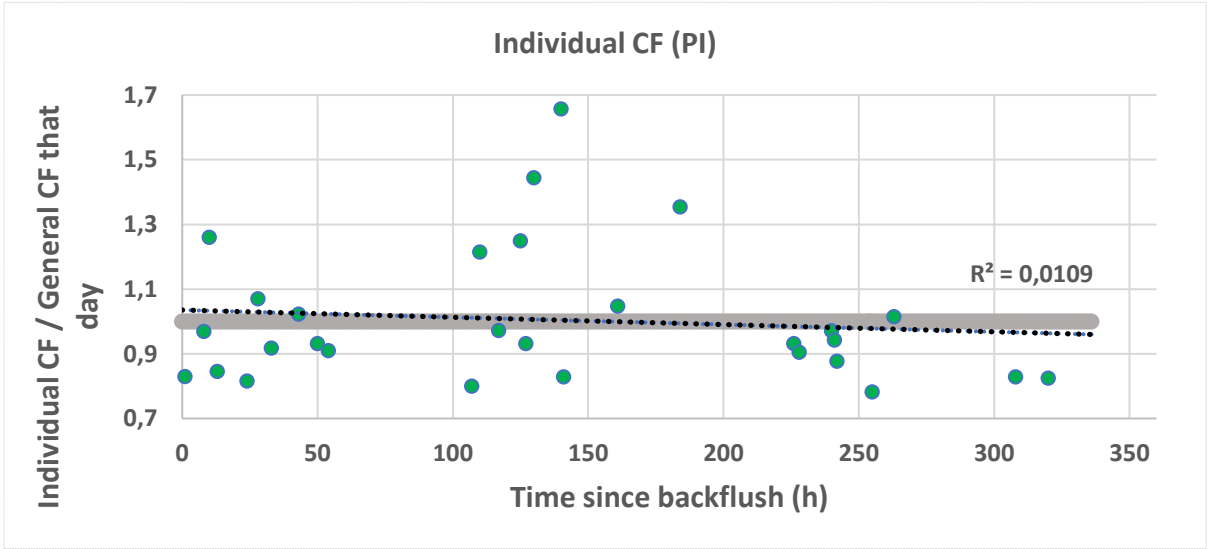
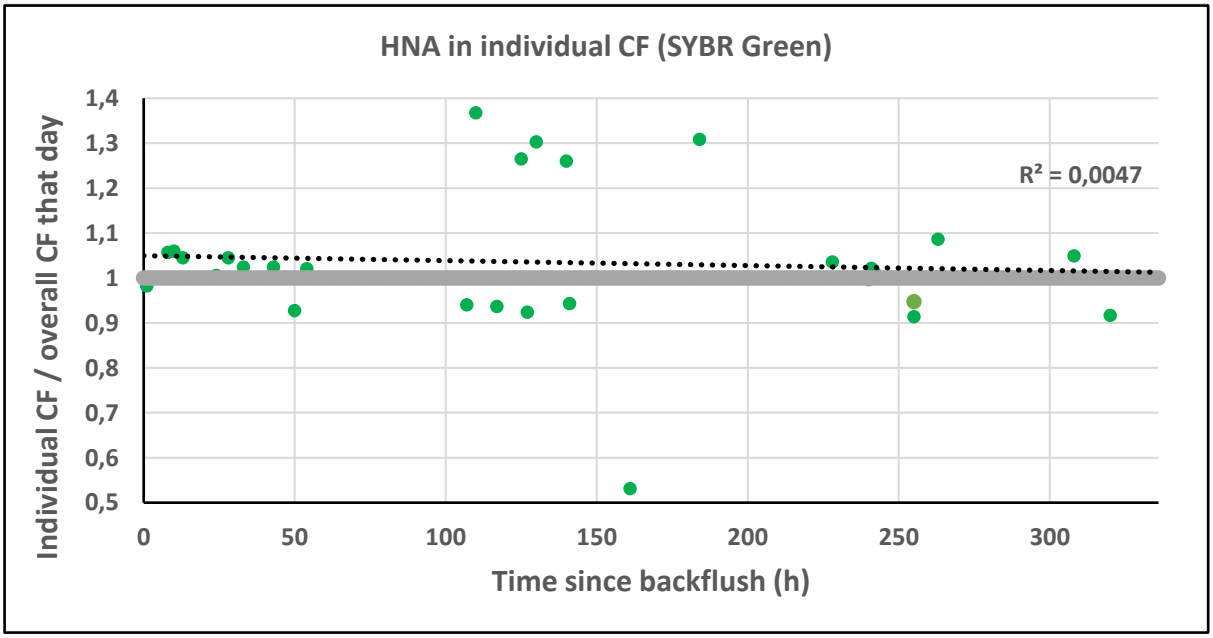


Figure 9: Bacterial counts in effluent sand filter water; individual sand filters compared to the average that specific date. The individual filter count is divided by the average, giving a ratio used for the Y-axis. Each dot is a mean value of triplicates. R^2 values are presented in each graph.


Individual CF (SYBR Green)
Individual CF / General CF that day
1,5
1,4
1,3
1,2
1,1
1
0,9
0,8
0,7
0
50
100
150
200
250
300
Time since backflush (h)
R² = 0,0134


Individual CF (PI)
Individual CF / General CF that day
1,7
1,5
1,3
1,1
0,9
0,7
0
50
100
150
200
250
300
350
Time since backflush (h)
R² = 0,0109


HNA in individual CF (SYBR Green)
Individual CF / overall CF that day
1,4
1,3
1,2
1,1
1
0,9
0,8
0,7
0,6
0,5
0
50
100
150
200
250
300
Time since backflush (h)
R² = 0,0047

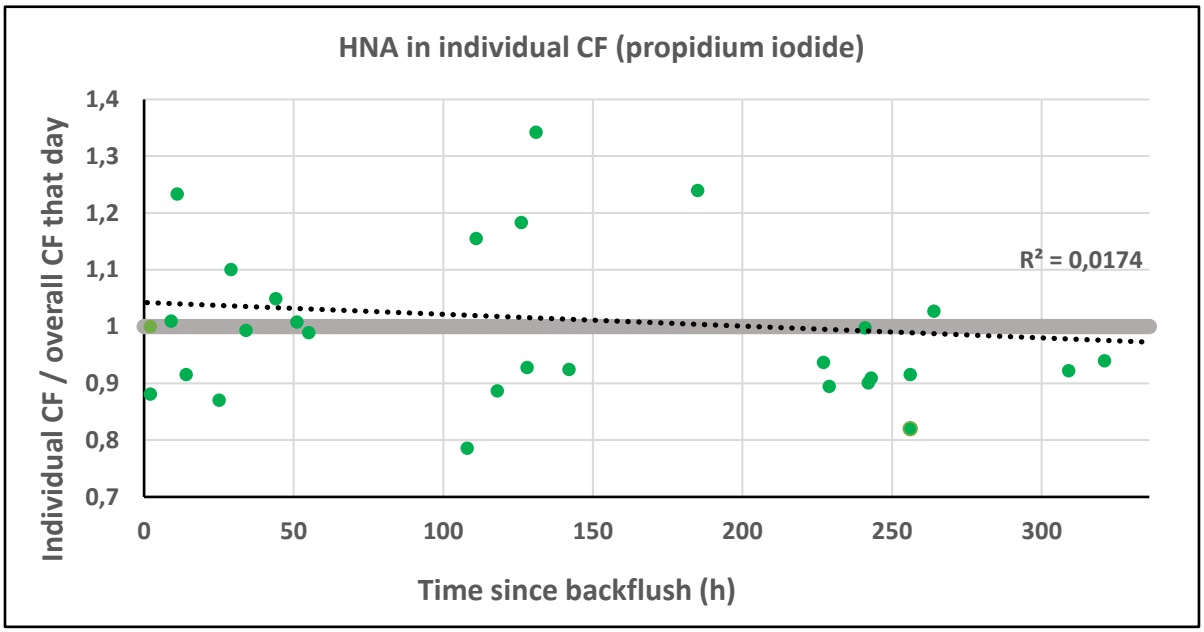


Figure 10: Bacterial counts in effluent carbon filter water, individual carbon filters compared to the average that date. The individual filter count is divided by the average, giving a ratio used for the Y-axis. Each dot is a mean value of triplicates. R^2 values are presented in each graph.

Total and intact bacterial counts for SF and R are shown in Figure 11. As mentioned, the treatment step in between them is precipitation + sedimentation or pulsator that have the same purpose. Here they are compared to each other. The bar graph also show reduction of bacteria for these steps of the treatment process. It shows that water passing the pulsator has slightly lower intact cell count in comparison between precipitation + sedimentation and pulsator (t = 0.0596). Total cell count is not significant in this way (t = 0.0196). It is important to note that the t-tests were performed on values from only 3 different days. More trials are required in order to obtain a more accurate comparison of the effectiveness of the pulsator and precipitation + sedimentation tanks.

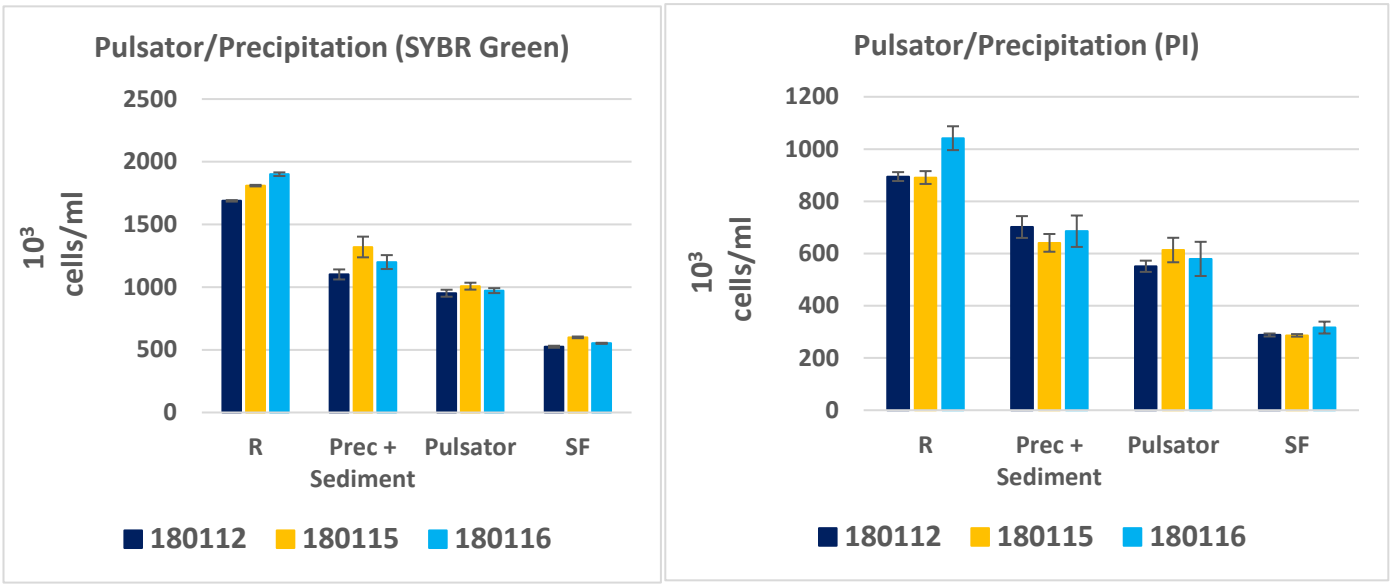


Figure 11: Bacterial separation efficiency of the two flocculation steps and sand filtration. Total and intact cell counts for treatment steps Precipitation + Sedimentation and Pulsator compared to before (R) and after sand filtration.

The effect of UV and monochloramine are shown in Figure 12. These treatments are crucial steps in the process to maintain drinking water quality during distribution. CF cells/ml increased significantly in all aspects after incubation at 4°C after the 16 days. Intact cell count was approximately doubled. Monochloramine addition lead to stable values or decrease. CF with UV treatment displayed fairly stable values in intact cell counts (around 270'000 cells/ml). Monochloramine addition [0.23 mg/l water] drives the intact cell count towards zero.

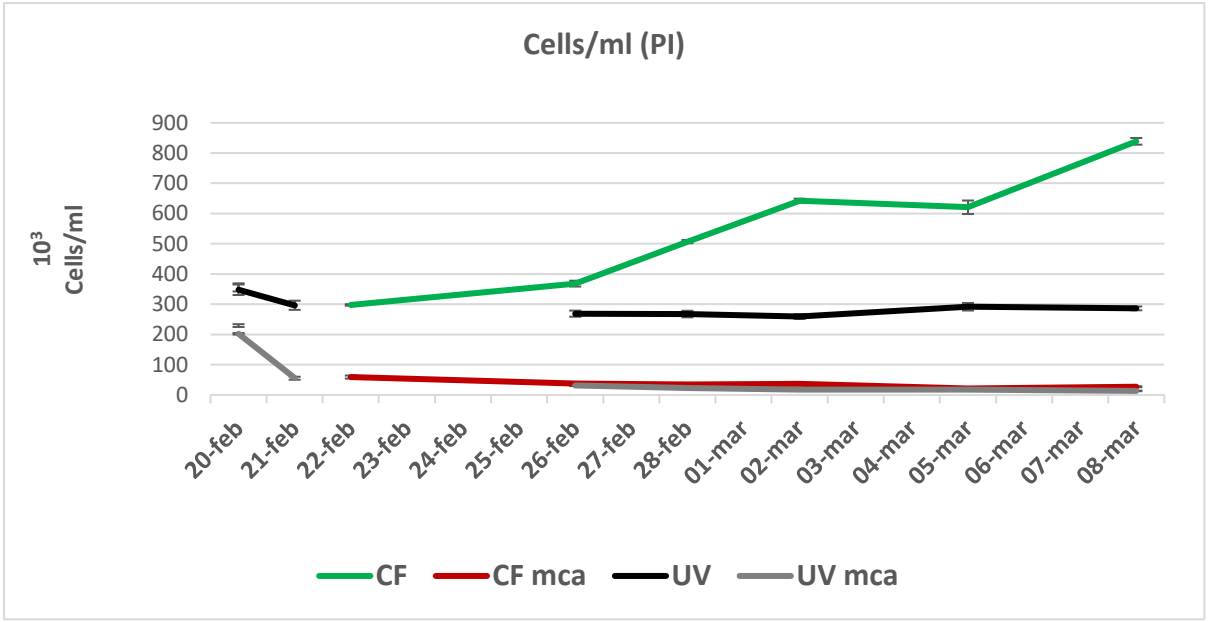
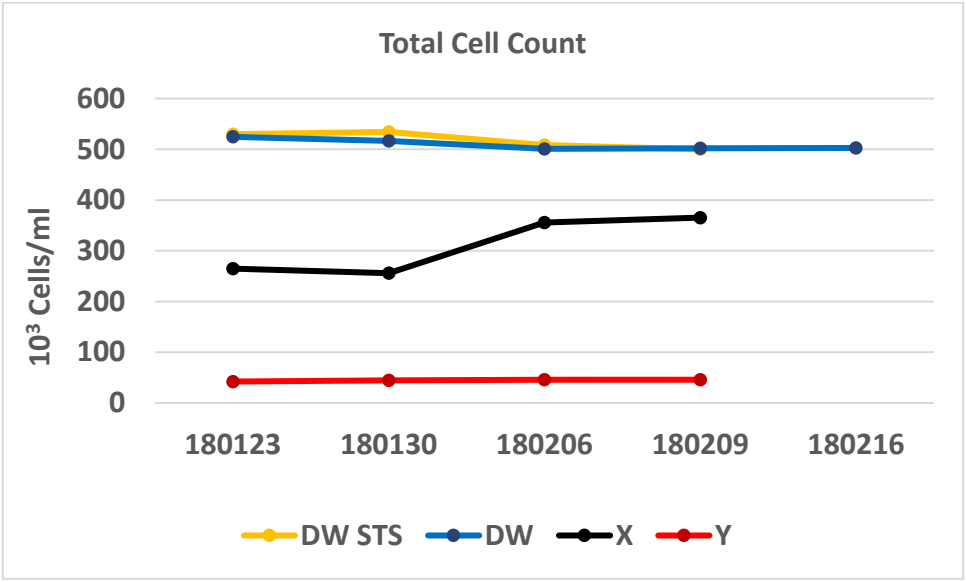


Figure 12: 16 days progression of intact cells/ml in closed glass bottles kept in 4°C. Carbon filtrated water +/- monochloramine and UV-treated carbon filtrated water +/- monochloramine.

Produced drinking water with and without STS was compared to two types of bought bottled water. The results over time are shown in Figure 13. Total cell count is fairly stable for 3 types of water, bottled water X seem to be increasing. Bottled water Y contains much less cells/ml than the other types. The same patterns can be seen using propidium iodide with one large difference, DW approaches zero.



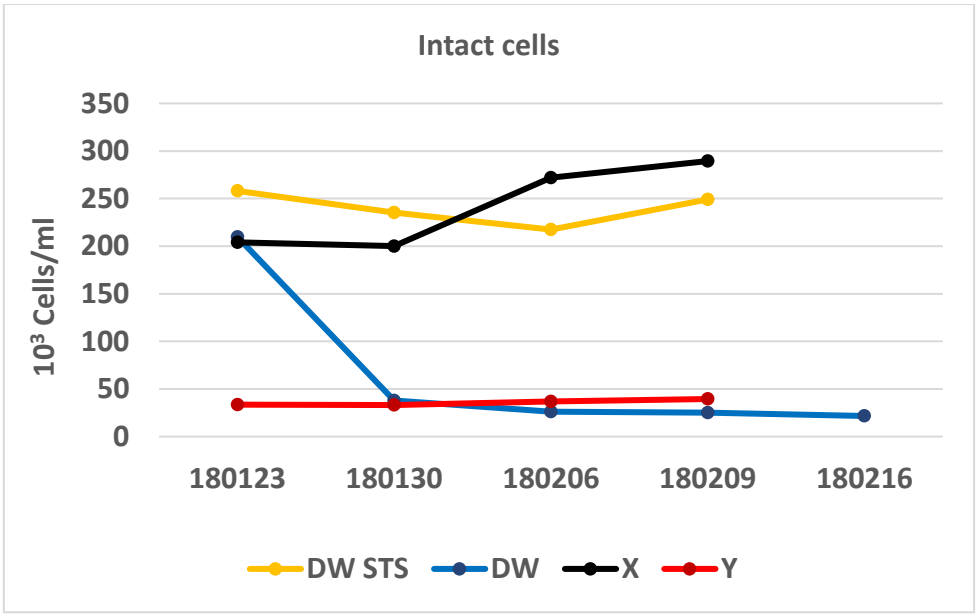


Figure 13: 24 days progression of total and intact cells/ml. Produced Drinking Water +/- STS compared to 2 types of bottled water. Samples were stored in closed bottles in 4°C.

Discussion

Heterotrophic plate counts show an apparently very low correlation with flow cytometric data. The best correlation can be seen in 3-days plate counts where the values for CF and SF stabilize at the end of December.

Raw water directly from lake Mälaren contains approximately 3 times the number of bacteria/ml compared to the three samples that have been processed that show similar values. The amount of HNA bacteria is more than four times higher in the raw water. The flocculation step removes a large proportion of bacteria (R to SF, visualized in Figure 11). After sand filtration the number of bacteria seem to be constant until final distribution of drinking water. This is explained by that the carbon filters do not remove bacteria. It is plausible that carbon filters have an indirect role on microbes by removing organic material from the water. Monochloramine and UV does not affect flow cytometric data instantly. It seems like these treatments require hours or days according to Figure 12 and 13. Samples were run shortly after collection, which explains why there is no significant difference in TCC/intact cell count between CF and DW.

Total- and intact bacterial levels decreased from October until it reached a fairly stable state after December as shown in Figure 2 and 3. Deviating slightly from this pattern is a decrease in counts for raw water when the intake conduit is switched at February 14th. The temperature of raw water covaries with the amount of sunlight and seems to be correlating well with the decrease of bacteria according to Figure 4. Stabilisation of counts coincides with the start of a HNA increase at the end of December when lake temperature is approximately 3.7 °C. It is plausible that water in lake Mälaren went through extensive circulation at this period that was caused by wind when surface water was the same temperature as at the bottom, when the thermocline is deformed (Brönmark & Hansson 2017). If so, bacteria from the lake sediment and surface may continuously go through the intake conduit. The increase in HNA may therefore be represented by sediment bacteria. Because HNA bacteria are generally the cultivable category, an increase should be seen in the heterotrophic plate counting, but that was not the case. It is therefore reasonable to assume that the bacteria are anaerobic with high nucleic acid content, not able to grow under aerobic heterotrophic plate counting conditions.

Investigating individual sand filters based on time since last back flush revealed several significant correlations. Total cell count, intact cell count and number of HNA bacteria all decreased the longer the filter was in progress. The reason for a decrease in values is likely to be due to biofilm formation in the sand filter, causing more and more bacteria to be stopped. For the same reason, back flushing becomes necessary because of mechanic clogging of the sand filter. Shortly after backflush, the bacterial cell count values are higher than the average of all filters. It is plausible that effluent water from sand filters shortly after backflush contain

biofilm bacteria. Another reason could be a partly disrupted biofilm, enabling more bacteria to pass through the filter. An accumulation of flocks and biofilm after backflush may improve filtration properties. After 30 hours, which is the half time point of flushing, the values are logically equal to the average of all filters. HNA bacteria measured with SYBR Green did not display this pattern, suggesting this data is not as trustworthy.

The main purpose of carbon filters is to provide a large active carbon surface for contact with passing water. Although the mechanism is not totally understood, it absorbs odorous compounds like geosmin produced by soil bacteria like Streptomyces among many. According to this experiment, carbon filters provide no significant decrease in total cell count, intact cell count or reduction of HNA bacteria. However, microbes are likely to exist in the carbon filters. This makes the drinking water production more complex to understand as there might occur microbial interactions.

The effects of UV and monochloramine were tested and results are shown in Figure 12. CF water seems to double the number of total and intact bacteria over the short experimental time, this applies to HNA as well (Figure 19). This indicates that CF water may not be treated enough to be sufficient drinking water after distribution. Not only regrowth of microbes is a reason to treat the water further than sand- and carbon filtration: parasitic protozoa are able to pass these barriers (Ottoson et al 2006). UV radiation creates an overall stable bacterial population unable to increase in numbers. Monochloramine had a radical effect on water types measured with propidium iodide. This is explained by the mechanism of chlorine compounds, which is to disrupt cell membranes by oxidation, which is the factor measured with propidium iodide. SYBR Green measurements of monochloramine treated water is therefore not as radical because nucleic acids are more or less still within the disrupted cell. Based on these data, monochloramine addition is a powerful disinfectant. However, it is important to understand the downsides before applying it in a water work. It may cause chemical reactions to form harmful or unpleasant compounds. Disrupting membranes of cells can release organic compounds usable as nutrients for potentially pathogenic organisms, enabling bacterial regrowth in the case of chlorine-resistance. It may be beneficial to conduct continuous measurements of dissolved organic carbon to assess microbial risks.

Two random bottled still waters were chosen as comparison to Norrvatten’s produced drinking water. Different characteristics can be seen in Figure 13. Bottled water Y contained fewer bacteria than Norrvatten’s and bottled water X. Perhaps Y is pumped from a groundwater source that underwent extensive natural filtration. Although numbers of bacteria are fewer in Y, it is important to point out that the proportion of HNA bacteria is much higher than the other water types (data not presented). Heterotrophic plate counts of bottled water showed more cfu than Norrvatten’s production (data not presented). In the water type called DW there is no STS present and therefore monochloramine affecting the water. This is clear in the propidium iodide staining where the number of bacteria approach zero. Such a long-term effect of monochloramine is beneficial during storage in water reservoirs to avoid bacterial regrowth. Biostability of the water types differ, the produced drinking water did not

show significant regrowth in total-, intact cell count or HNA (Figure 13 and 20). Importantly, produced drinking water containing STS yielded massive growth as determined by heterotrophic plate counting and 14 days of incubation at 4 °C (data not presented). Monochloramine was highly effective and created satisfactory biostability. Bottled water Y showed no sign of increasing bacterial numbers throughout the 17 days of measurements. Bottled water X differed from the other two, showing increased counts in all 3 aspects. X also yielded by far the most cfu as by heterotrophic plate counting, Y second and DW usually no cfu (data not presented).

Conclusions

- Total and intact bacterial cell counts in lake Mälaren start high in October and decline until the end of December. Counts are then stable until March.
- HNA counts are lowest when total and intact cell counts reach their stable levels. HNA then increases when temperature is around 4°C. The reason may be anaerobic sediment bacteria rising to the intake conduit due to lake mixing.
- The two alternative paths, flocking + sedimentation chamber and pulsator, were compared. The pulsator seem more effective in removing intact cells although more runs are needed for statistics.
- Carbon filters do not alter total, intact or HNA bacterial counts.
- Sand filtration effluent water had the highest cell counts in the beginning of its operating time of 60 hours. Counts then decreased steadily until backflush. The reason may be a disrupted biofilm with its bacteria going through the filter after backflush. Later, the effective biofilm is once again established, reducing the number of passing bacteria.
- Drinking water produced by Norrvatten rarely yield any cfu as determined by heterotrophic plate counting. Biostability is sufficient because of monochloramine addition and UV radiation, which is not the case in to varieties of commercial bottled water.

Acknowledgements

I would like to thank Norrvatten with employees for allowing me to conduct this master's thesis project using their facilities, instruments and available chemical data. Most importantly I would like to thank my dedicated supervisor at Norrvatten, Linda Holmer, who taught me flow cytometry and everything needed to work in the laboratory. This study was planned in collaboration with Linda whose great insight into the water works provided numerous interesting aspects to study. Linda also assisted in my understanding of data management. I would like to thank my academic supervisor, Peter Rådström, for providing great feedback on drafts of the project report. Lastly, I thank graphic- and interaction designer, Melinda Danielsson, for creating the front page.

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Supplementary information

Chemical precipitation

These treatment steps consist of flocculation, sedimentation and filtration (Madigan et al 2012). This is necessary to remove humus, inorganic particles and algae too small for the initial filter and sedimentation. These compounds are generally negatively charged, causing them to repel and prevent spontaneous flocculation. A precipitation chemical like aluminium sulphate is necessary to neutralize charge to make compounds flock (Svenskt Vatten 2010). Sodium silicate can be added to increase the size of flocculates and speed up the process of sedimentation (Norrvatten). In order for this to work, important aspects to consider are pH and concentration of precipitation chemical (Svenskt Vatten 2011). Norrvatten applies 2 separate techniques in this step of the process. The first technique uses the method described above in 2 different tanks. The second technique is the pulsator facility (Degrémont) that combines flocking and sedimentation in the same tank. Instead of mechanical stirring, water is moved by vacuum suction and taken care of by a distribution system that in the end separates sludge into a sludge concentrator (Svenskt Vatten 2010).

Filtration

Chemical precipitation and sedimentation is followed by sand filtration. This removes remaining flocks, bacteria, parasites and virus (Svenskt Vatten 2011). There are 18 individual sand filters and they are approximately 1.2 meter thick with the finest grains on top (Norrvatten). When the turbidity of passing water increases, maintenance such as backflushing is initiated. The time when filters are active depends on the material's capacity of storing flocks etc and the quality of incoming water. During the experimental time, Norrvatten applied 60 hours between backflush as standard. After backflush, the first 15 – 30 minutes of passing water is usually redirected straight to sewers and not used for further processing. After sand filtration, the water passes through carbon filters. The active carbon has an extremely high surface area for chemical absorptions. Each gram of carbon has more than 1000 m^2 of surface and each pore is 5 – 100 Å wide (Svenskt Vatten 2010).

UV radiation

After carbon filtration, the water passes through UV radiation. UV form pyrimidine dimers in the DNA of bacteria. Bacterial DNA polymerase cannot pass such damaged DNA accurately due to steric prevention in the enzyme's active site. In this way, deletion- and insertion mutations occur (Craig et al 2014). A great benefit of UV treatment is effectiveness against chlorine-tolerant organisms like *Cryptosporidium*, even cysts. Unfortunately, pathogens possess DNA repairing mechanisms that might enable them to recover after UV radiation if damage is not too serious (Madigan et al 2012). If UV is applied in drinking water treatment, it is important to apply it in the end because higher levels of humus interferes with UV efficiency to reach cells (Svenskt Vatten 2010).

pH adjustment and monochloramine addition

Treated water is slightly adjusted with agricultural lime to a pH that does not cause corrosion on distribution pipes. UV radiation can be combined well with chlorination as final steps in drinking water production. Monochloramine is added to a final concentration of approximately 0.23 mg/l. It inactivates through oxidative disinfection and is therefore not a separating barrier like sand filtration. Chloramine is a weak oxidant and therefore not considered a microbiological barrier in drinking water production. It is used in the end of water processing to avoid development of taste- and odorous compounds for aesthetic reasons as well as preventing biofilm formation in distribution systems. It is highly effective on bacteria although some exceptions are sporulating bacteria like *Bacillus* and *Clostridium spp* (Svenkt Vatten 2011). Chloramine can diminish in the distribution system with microbial regrowth as a consequence (Hoefel et al 2005). Drinking water quality is then considered to be degraded and the risk for odorous compounds increases (Waller et al 2018). Flow cytometry can detect such a bacterial regrowth along the distribution line (Hoefel et al 2005). The process of chlorination does not remove any organic matter, instead it oxidizes it, creating more easily obtainable nutrients like carbon for remaining microbes (Liu et al 2013a; van der Wielen & van der Kooij 2010; Svenskt Vatten 2011; Svenskt Vatten 2010). Microbial regrowth is significantly decreased if organic nutrients are removed with nano- or ultrafiltration (Liu et al 2013a). Furthermore, there are highly chlorine tolerant organisms like the parasitic protozoa *Cryptosporidium parvum* and *Giardia intestinalis* that have caused several serious outbreaks worldwide (WHO 2015; Svenskt Vatten 2011). Chlorination is applied as a final step in drinking water processing because the turbidity due to organic material is relatively low, which means minimal protection of microbes from particles. Furthermore, chlorine can react with compounds like phenol still present in the water, creating trihalomethanes which are odorous and harmful (Svenskt Vatten 2011; Svenskt Vatten 2010).

Water reservoirs

Finished water is distributed to customers or stored in reservoirs. Norrvatten owns eight water reservoirs that are useful to ensure continuous distribution of the final product to customers. Water from reservoirs may differ from those delivered straight from the water works. Multiple actions are made in the water works to inhibit regrowth of bacteria in reservoirs: Ultraviolet light radiation and addition of monochloramine. Ideally, these two actions ensure high quality of the product even after storage time that would otherwise enable regrowth of potentially harmful bacteria. Produced drinking water may have to be stored up to 2 weeks before reaching customers (Norrvatten). Figure 18 shows flow cytometric data from the water reservoirs Ensta water tower and Oxbergsreservoaren. Samples were obtained once every month from November 16th until February 15th.

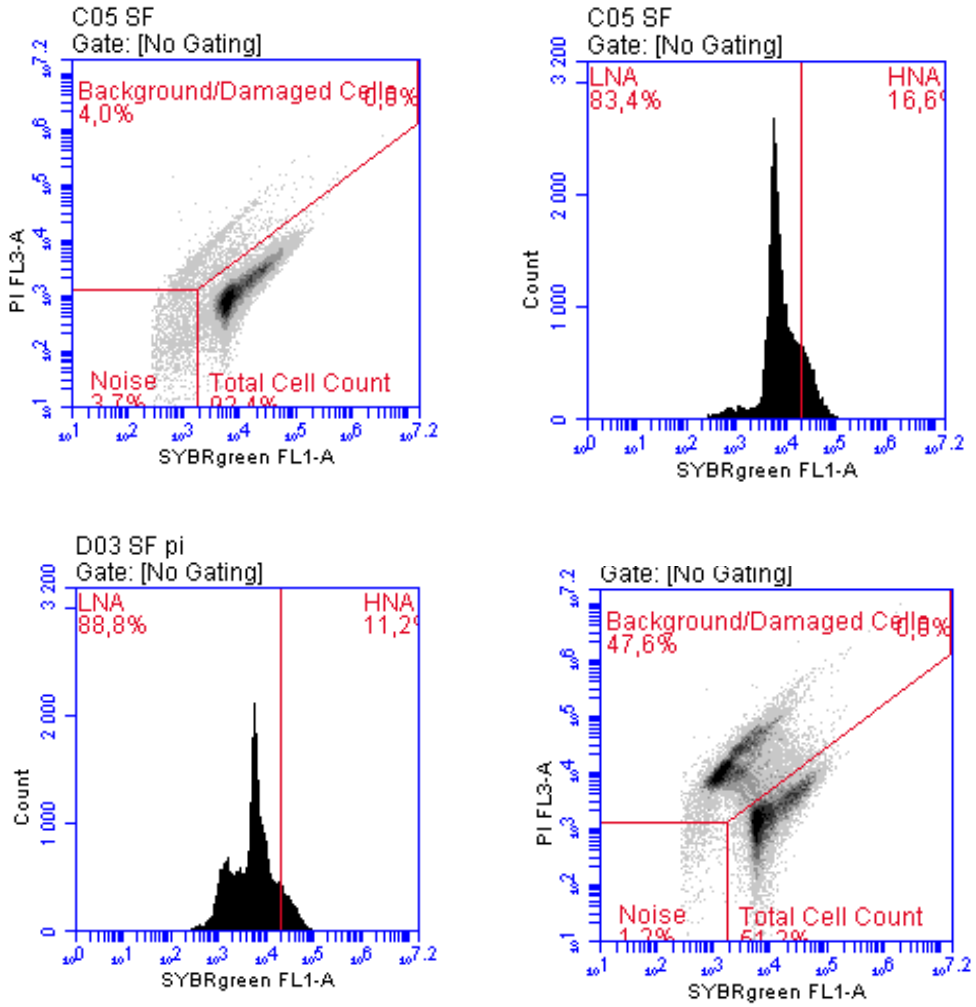


Figure 14: Raw flow cytometric data. Dot-plots with the gate of choice and separation of HNA/LNA according to Hammes et al 2008. Top left shows data collected by using SYBR Green staining, bottom right shows the same sample using propidium iodide. Top right and bottom left show the artificial standardized HNA/LNA threshold.

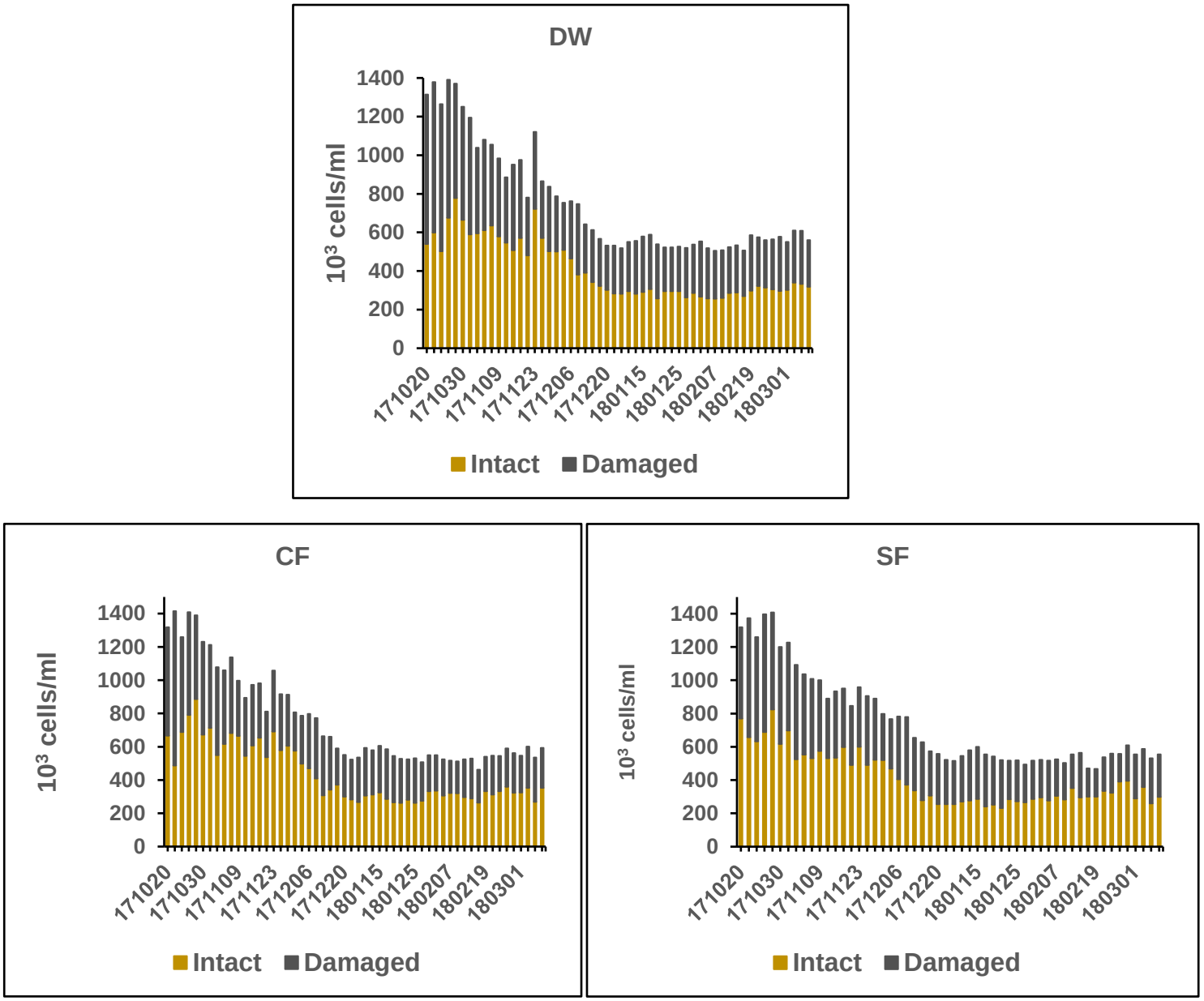


Figure 15: Proportions of intact and total cell counts in 3 routinely analysed processed samples throughout the study.

Daily fluctuations of DW (January 31st) and CF (February 19th) total and intact cell counts were measured with flow cytometry. This analysis is useful to show if time of sampling during a day is significant. These data are presented in Figure 16 and 17. Mean values of total cell count in DW varied between 495'000 and 542'000 (9 %). Intact cell count varied between 242'000 and 286'000 (15 %). CF varied 521'000 – 558'000 (7 %) and 301'000 – 368'000 (18 %) respectively. HNA cells varied <1% among all the measurements (data not presented).

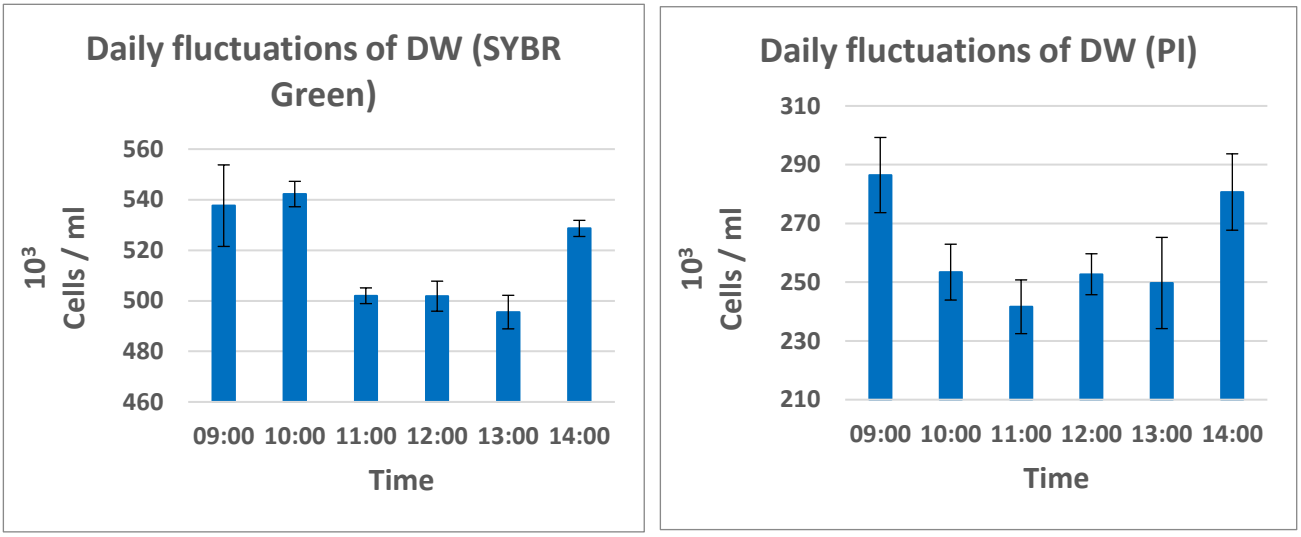


Figure 16: Daily fluctuations of bacterial counts in DW measured flow cytometrically with SYBR Green and propidium iodide staining.

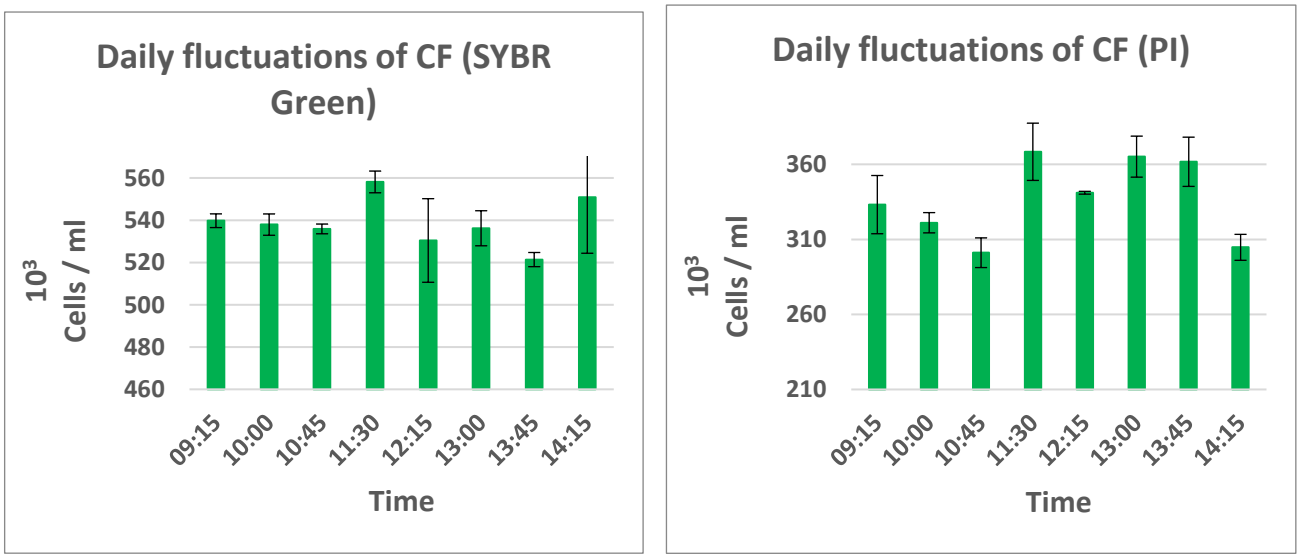
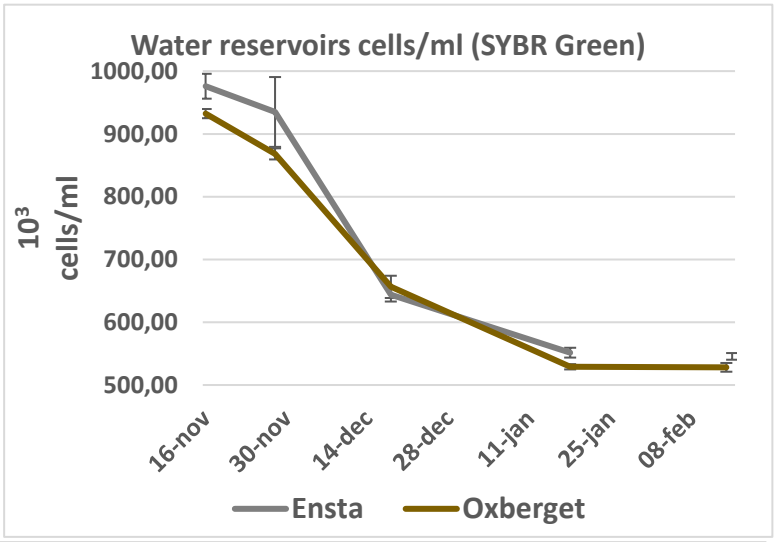
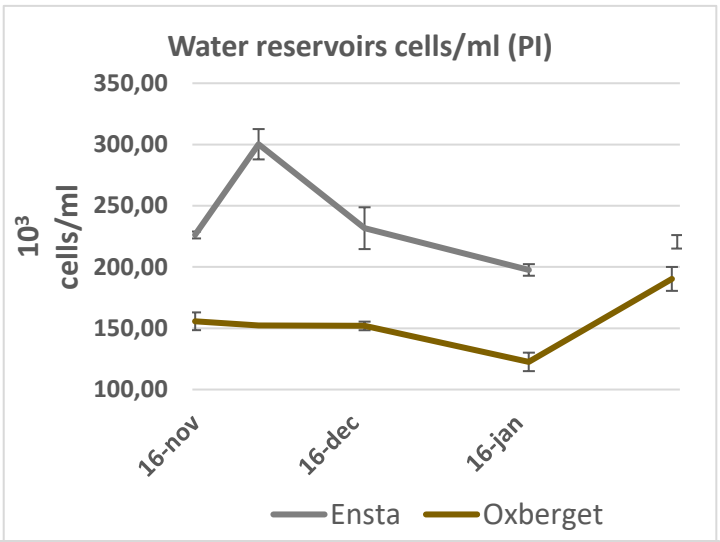
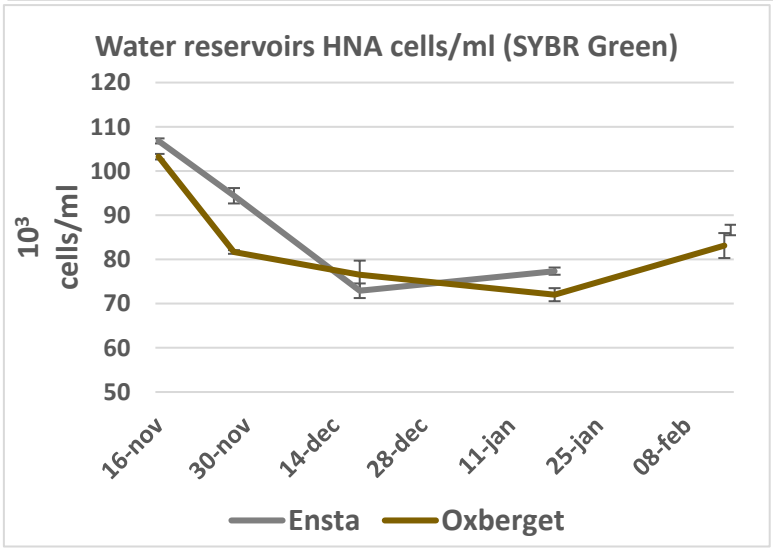


Figure 17: Daily fluctuations of bacterial counts in CF measured flow cytometrically with SYBR Green and propidium iodide staining.







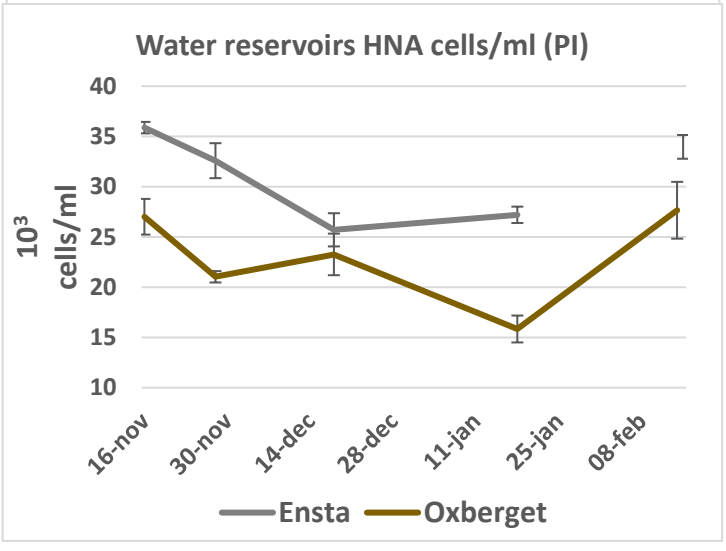
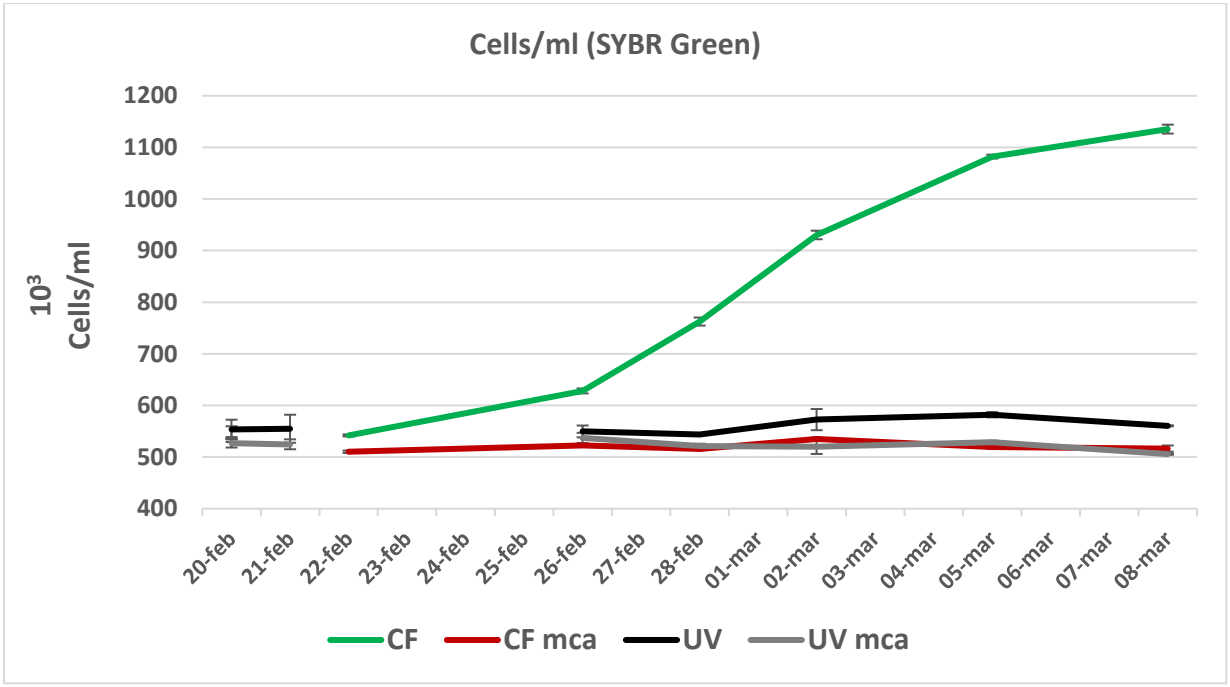


Figure 18: Two water reservoirs total- and intact cell counts and number of HNA bacteria. Samples are taken approximately twice every month.


Cells/ml (SYBR Green)
10³ Cells/ml
1200
1100
1000
900
800
700
600
500
400
20-feb
21-feb
22-feb
23-feb
24-feb
25-feb
26-feb
27-feb
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CF
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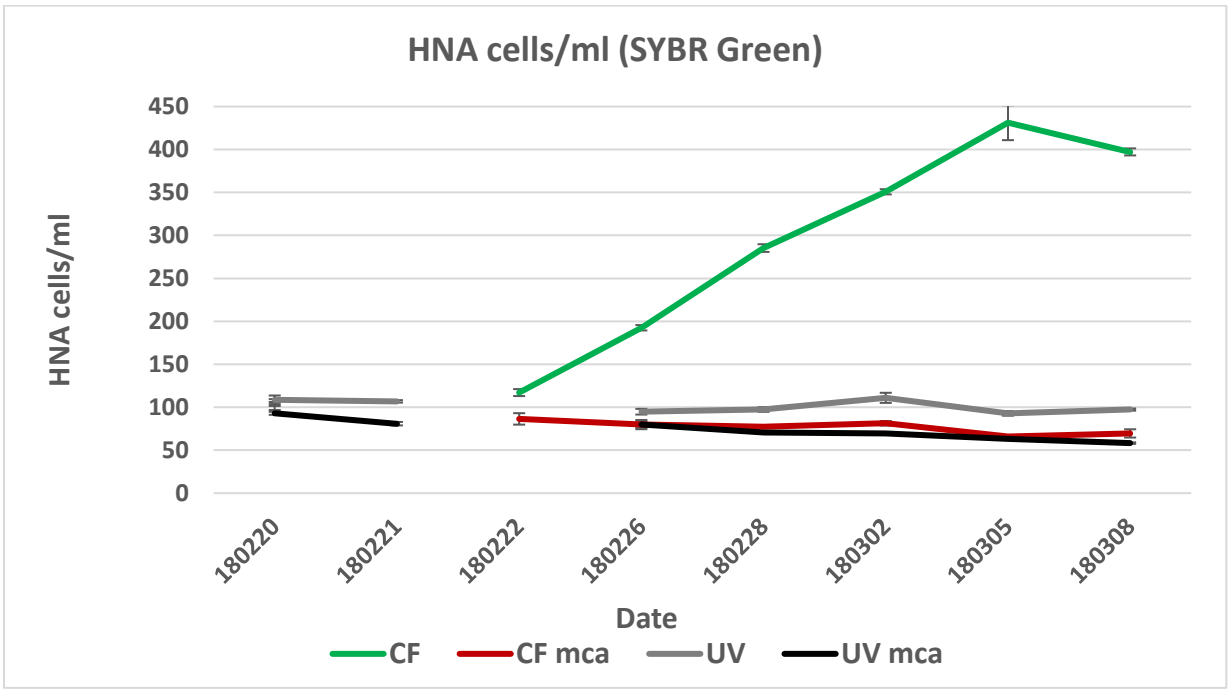
HNA cells/ml (SYBR Green)
HNA cells/ml
450
400
350
300
250
200
150
100
50
0
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180221
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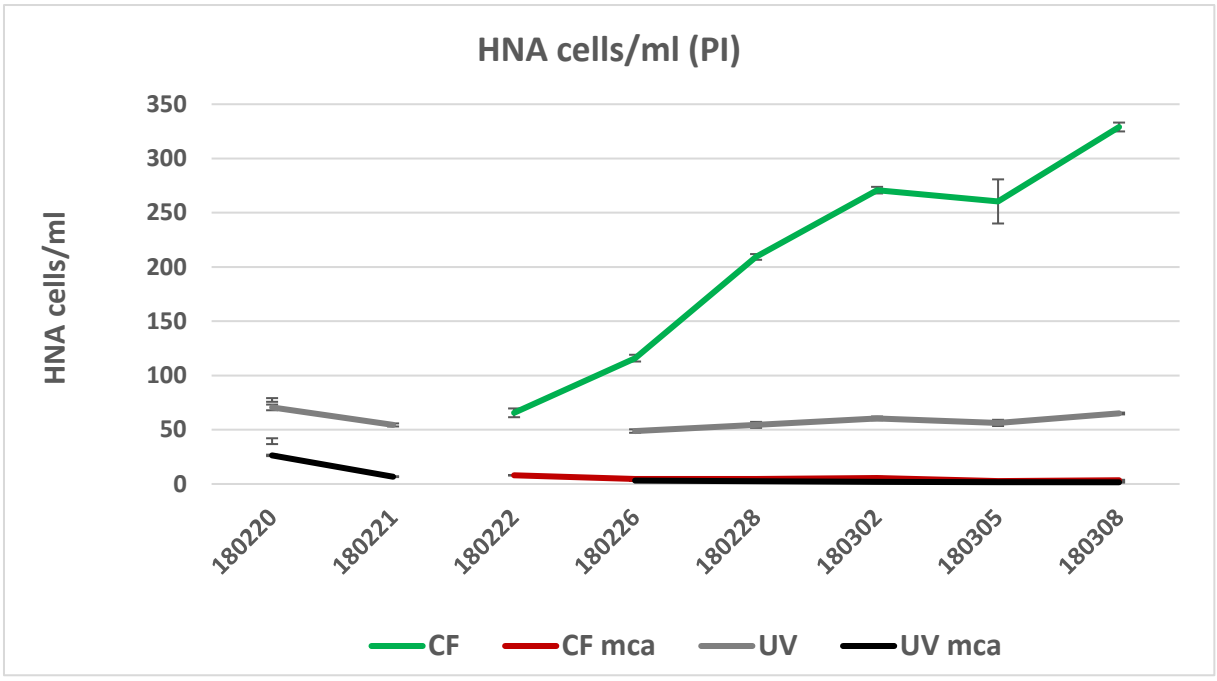

Figure 19: 16 days progression of HNA, total and intact cells/ml in closed glass bottles kept in 4°C. Carbon filtrated water +/- monochloramine and UV-treated carbon filtrated water +/- monochloramine. Error bars show standard deviation among triplicates.

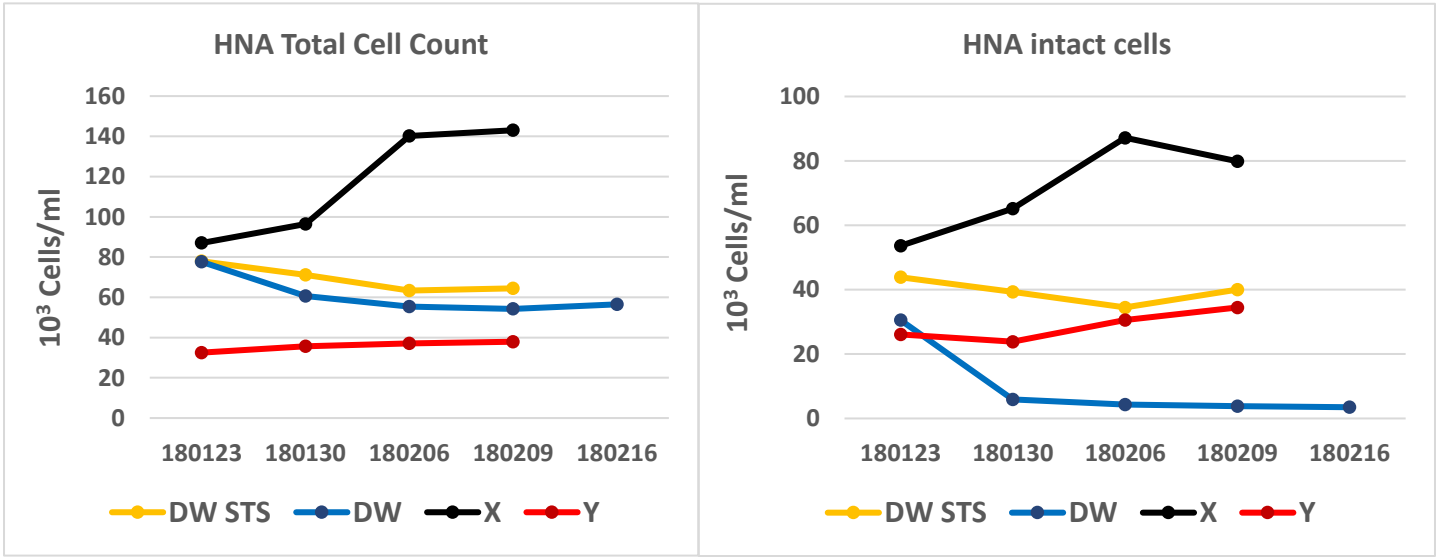

Figure 20: Produced Drinking Water +/- STS compared to 2 types of bottled water. 24 days of storing in closed bottles in 4°C.